REMARKS

Reconsideration and withdrawal of the rejections of the claims, in view of the amendments and remarks herein, is respectfully requested.

Claims 41, 43-44 are amended, claims 1-9, 17-40, 42, 45, 53-56, and 65-68 are canceled, and claims 77-82 are added; as a result, claims 10-16, 41, 43-44, 46-52, 57-64, and 69-82 are now pending in this application. Claims 10-16, 41, and 48-52 are previously presented.

Support for the amendment to claims 43-44 is found in the specification at page 12, line 32-page 13, line 15; page 18, lines 15-27; page 42, lines 10-21 and Example 5.

Support for new claims 77-79 is found in the specification at page 34, lines 14-17 and page 35, line 30-page 36, line 1.

Support for new claims 80-81 is found in the specification at page 12, line 32-page 13, line 15; page 18, lines 15-27; page 42, lines 10-21 and Example 5.

Support for new claim 82 is found in the specification at page 2, lines 6-11; page 8, lines13-21; page 17, line 30-page 18, line 27; page 34, lines 14-17; page 35, line 32-page 36, line 1; and page 49, lines 19-22.

The present invention

The peripheral-type benzodiazepine receptor (PBR) is an 18-kDa protein. It is expressed at particularly high levels in steroid-producing tissues, where it is localized primarily in the outer mitochondrial membrane (OMM) (see page 1, line 33-page 2, line 2 of the specification). PBR is a functional component of the steroidogenic machinery, and mediates cholesterol delivery from the OMM to the inner mitochondrial membrane (IMM) (see page 2, lines 7-11 of the application). The rate-determining step in hormone-stimulated steroid biosynthesis is the transport of cholesterol from intracellular, extramitochondrial sources to the IMM. The present invention is based upon the discovery of several PBR-associated proteins (PAPs) that interact with PBR, including one with high affinity for PBR, *i.e.*, PAP7. PAPs play a role in proper targeting, function, expression and/or stability of PBR (page 34, lines 14-17 of the specification).

The rejected claims are directed to a nucleic acid sequence that is at least 90% identical to a nucleotide sequence encoding SEQ ID NO:7, which nucleic acid sequence encodes a polypeptide that is capable of regulating progesterone biosynthesis, or the complement thereof

(claim 43), to such a nucleic acid sequence that encodes a polypeptide that impairs cholesterol delivery, or the complement thereof (claim 44), and to such a nucleic acid sequence that encodes a polypeptide that increases cholesterol delivery, or a complement thereof (claim 77); an isolated nucleic acid comprising a nucleic acid sequence encoding SEQ ID NO:7 and variants thereof that are at least 90% identical to the nucleic acid sequence, which isolated nucleic acid encodes a polypeptide that facilitates cholesterol transport from the outer mitochondrial membrane to the inner mitochondrial membrane (claim 82); an isolated nucleic acid that encodes a polypeptide that is capable of regulating progesterone biosynthesis and hybridizes to the complement of SEQ ID NO:2 or a nucleotide sequence encoding SEQ ID NO:7 under the following stringent conditions: a final wash in 0.1X SSC at 65°C (claim 46); an isolated nucleic acid that encodes a polypeptide that is capable of impairing cholesterol delivery and hybridizes to the complement of SEQ ID NO:2 or a nucleotide sequence encoding SEQ ID NO:7 under the following stringent conditions: a final wash in 0.1X SSC at 65°C (claim 47); an isolated nucleic acid that encodes a polypeptide that is capable of increasing cholesterol delivery and hybridizes to the complement of SEQ ID NO:2 or a nucleotide sequence encoding SEQ ID NO:7 under the following stringent conditions: a final wash in 0.1X SSC at 65°C (claim 79); and a process of producing a PAP comprising culturing a host cell of the invention under suitable conditions to express a PAP7 encoded by the nucleic acid (claims 59, 63, 71 and 75).

The 35 U.S.C. § 112 Rejections

The New Matter Rejection

The Examiner rejected claims 44, 47, 61-64, and 73-76 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention (a new matter rejection). In particular, the Examiner assert that the specification as originally filed does not provide adequate written support for the phrase "impairs cholesterol delivery" in claims 44 and 47. This rejection is respectfully traversed.

The present application discloses that PAP7 interacts with PBR (page 5, lines 19-24; page 34, lines 14-17) and regulates PBR activity in cholesterol transport (page 49, lines 19-22). For

example, it is disclosed that the function or stability of PBR and associated PAP7 can be manipulated to increase cholesterol transport into cells (page 35, lines 32-page 36, line 10; page 50, lines 11-17), or, alternatively, PAP7 can be used to competitively bind PBR and block normal PBR function, *i.e.*, impair cholesterol transport into the IMM (page 51, lines 18-26).

Accordingly, withdrawal of the new matter rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

The Enablement Rejection

The Examiner rejected claims 43-44, 46-47, 57-64, and 69-76 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which is pertains, or with which it is most nearly connected, to make and/or use the invention. As this rejection may be maintained with respect to the pending claims, it is respectfully traversed.

Specifically, the Examiner alleges that the presently claimed invention is not enabled because (1) there is no nexus in the instant specification that pregnenolone reflects accumulation of cholesterol in the inner mitochondrial membrane (IMM); (2) undue experimentation would be required to generate and screen an isolated nucleic acid sequence that hybridizes to the complement of SEQ ID NO:2 or is 90% identical to SEQ ID NO:2 and encodes a polypeptide that is capable of regulating progesterone biosynthesis or that impairs cholesterol delivery, citing to Wuyts *et al.* (J. Immunol., 163:6155 (1999)), Sher *et al.* (J. Biol. Chem., 274: 35016 (1999)) and Kopchick *et al.* (U.S. Patent No. 5,350,836) to support the proposition that structurally similar proteins have different functions; and (3) the specification does not teach methods or working examples that indicate PAP7 mediates cholesterol delivery or regulates PBR activity in cholesterol delivery.

As for basis (1) of the rejection, the specification discloses that PBR ligands stimulate pregnenolone formation by increasing the rate of cholesterol transfer from the outer to the inner mitochondrial membrane (page 3, lines 10-23). Therefore, Applicants have described a nexus between pregnenolone and cholesterol accumulation in the IMM.

Regarding (2), it is Applicants' position that one of ordinary skill in the art in possession of Applicants' specification and knowledge generally available to the art would be apprised of

how to make and identify the claimed nucleic acid sequences. Applicants' detailed description discloses that PAPs may be identified by screening a mammalian cDNA library using PBR as bait in a yeast two-hybrid system (page 5, lines 19-22; page 11, line 10-page 12, line 31; page 40, lines 6-23 and Example 1). The present specification discloses the nucleotide and polypeptide sequences of PAP7 (SEQ ID NO:2 and SEQ ID NO:7, respectively), and how to make variants of PAP7. See, for example, page 17, line 30-page 18, line 27. In addition, the claimed sequences may be isolated from a genomic or mutated library using conventional molecular biology methodology, or chemically synthesized. Moreover, it is respectfully submitted that it is well within the skill of the art to determine whether or not a sequence has at least 90% identical to another by, for example, visual inspection or employing a computer algorithm such as is available on the world wide web at ncbi.nlm.nih.gov/BLAST/. Furthermore, it is submitted that one of ordinary skill in the art would be well-equipped to determine whether or not a claimed nucleic acid hybridizes to the complement of SEQ ID NO:2 or the complement of a nucleotide sequence encoding SEQ ID NO:7 under stringent conditions, *i.e.*, a final wash of 0.1X SSC at 65°C. See, for example, page 17, lines 11-18 of the specification and pages 9.47-9.62 of Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, 2nd edition (1989) (a copy is enclosed).

In addition, Example 5 of the application discloses how to determine the effect of a molecule, e.g., a PAP7-related molecule, on steroid biosynthesis. Moreover, the specification discloses that fragments of PAP7, which include the PBR binding domain, reduce the level of progesterone formation stimulated by saturating concentrations of hCG (50 ng/ml) compared to control (empty vector) cells (Example 5; page 51, lines 10-14). Thus, the PAP7 fragment is a *competitor* of native PAP7 in MA-10 cells, *i.e.*, it competitively binds to the PBR binding domain of PBR resulting in reduced cholesterol delivery to the IMM, thus regulating progesterone biosynthesis (page 51, lines 18-21).

Further, the Examiner is urged to consider that prior to Applicants' filing, the art worker was aware of assays to determine the ability of a claimed polypeptide to regulate progesterone biosynthesis and cholesterol delivery/transport. See, for example Papadopoulos *et al.*, The Journal of Biological Chemistry, 265:3772 (1990) (a copy is enclosed), who disclose steroid biosynthesis experiments, and Krueger and Papadopoulos, The Journal of Biological Chemistry,

265:15015 (1990) (a copy is enclosed), who disclose cholesterol translocation assays (note that each document is incorporated by reference at page 2, lines 8-12 of the present application).

The Federal Circuit has explicitly recognized that the need, and methodologies required, to carry out extensive synthesis and screening programs to locate biomolecules with particular properties do not constitute undue experimentation. In re Wands, 8 U.S.P.Q.2d 1400, 1406-1407 (Fed. Cir. 1988), the Court stated:

> The nature of monoclonal antibody terminology is that it involves screening hybridomas to determine which ones secrete antibody with desired characteristics. Practitioners of this art are prepared to screen negative hybridomas in order to find one that makes the desired antibody.

Likewise, practitioners in the art related to the present application would be well-equipped to prepare and/or screen for the claimed nucleic acids. See also, Hybritech Inc. v. Monoclonal Antibodies Inc., 231 U.S.P.Q. 81, 84 (Fed. Cir. 1986) (evidence that screening methods used to identify characteristics [of monoclonal antibodies] were available to art convincing of enablement). Thus, the fact that a given claim may encompass a variety of molecules is not dispositive of the enablement issue, particularly in an art area in which the level of skill is very high and in which screening of large numbers of compounds has been standard practice for at least ten years (Ex parte Forman, 230 U.S.P.Q.2d 456 (Bd. App. 1986).

In fact, the Examiner is urged to consider that both Sher *et al.* and Kopchick *et al.* provide evidence that it was well within the skill of the art worker, as of the filing date of the present application, to make and screen variant polypeptides.

Thus, given Applicants' disclosure, and the skill of the art worker in the relevant art area, it is respectfully submitted that it would not require undue experimentation for the art worker to prepare and screen isolated nucleic acids of the invention.

Sher *et al.* report the biological activity and receptor binding of mutant fibroblast growth factor 7 (FGF-7) proteins, which mutants included individual amino acid substitutions in a loop domain and a chimeric FGF-7 protein having a corresponding loop from FGF-2 (page 35017, left column). Note that many of the tested mutants in Sher *et al.* retained at least some activity. Kopchick *et al.* disclose the activity of mutant growth hormones having an alteration in the third alpha helix. The mutants fell into four groups, three of which had either agonist or antagonist activity (of 20 mutants in Tables I-IV, only 2 double mutants were "non-functional"). Wuyts *et*

al. disclose that naturally occurring NH_2-terminal and COOH-terminally truncated forms of murine granulocyte chemotactic protein-2, a CXC chemokine, augmented chemotactic potency for human and murine neutrophils (abstract). Thus, none of the cited art provides evidence that generally variant proteins are inactive, *i.e.*, can tolerate no or only conservative substitutions.

Moreover, "the 'predictability or lack thereof' in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed invention" (M.P.E.P. § 2164.03). Accordingly, in view of Applicants' specification, one of ordinary skill in the art would find it reasonably predictable to prepare the claimed PAP7 nucleic acid sequences, *e.g.*, those which encode proteins that regulate progesterone synthesis and/or cholesterol delivery.

Regarding basis (3) of the rejection, it is Applicants' position that, as discussed above, the specification clearly discloses that PAP7 mediates cholesterol delivery, for example, by competitively binding native PAP7 and impairing delivery of cholesterol from the OMM to the IMM, or, for example by stabilizing PBR and increasing cholesterol transport across the mitochondrial membrane (see page 34, lines 15-17 of the specification). Moreover, the Examiner is respectfully reminded that there is no requirement for a working example to fulfill the requirement of § 112(1). In re Robins, 166 U.S.P.Q. 552 (C.C.P.A. 1970); In re Borowski, 422 F.2d 904, 164 U.S.P.Q. 642 (C.C.P.A. 1970). If Applicant's invention is disclosed so that one of ordinary skill in the art can practice the claimed invention, even if the practice would include routine screening or some experimentation, Applicant has complied with the requirements of § 112(1). In re Angstadt, 190 U.S.P.Q. 214 (C.C.P.A. 1976); Ex parte Jackson, 217 U.S.P.Q. 804 (Bd. Appl 1982).

Hence, Applicants' specification is in compliance with the enablement requirement of § 112(1).

The Written Description Rejection

The Examiner rejected claims 43-44, 46-47, 57-64, and 69-76 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. As this rejection may be maintained with respect to the pending claims, it is respectfully traversed.

In particular, the Examiner asserts that Applicants have not provided evidence to demonstrate the skilled artisan would be able to envision the detailed structure of the infinite number of polynucleotides recited in the claims, and that the description of one PAP7 polynucleotide and its encoded polypeptide in the specification is not a representative number of embodiments to support the description of an entire genus (page 10 of the Office Action).

The function of the written description requirement is to ensure that a patent is granted to inventors who had possession, as of the filing date of the application relied on, of the specific subject matter later claimed by them; how the specification accomplishes this is not material. In re Smith, 178 U.S.P.Q. 620 (C.C.P.A. 1973). Therefore, the test for written description under 35 U.S.C. §112, first paragraph, is whether the originally filed specification reasonably conveys to a person having ordinary skill in the art that Applicants had possession of the subject matter later claimed. M.P.E.P. § 2163.02. See also, In re Kaslow, 217 U.S.P.Q. 1089 (Fed. Cir. 1983). What is conventional or well known to one of ordinary skill in the art need not disclose in detail. M.P.E.P. § 2163.II.A.3 (a) (citing to Hybritech Inc. v. Monoclonal Antibodies, Inc. 802 F2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986)).

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice . . . or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶1, "Written Description" Requirement, Federal Register, 66, 1099, 1106 (January 5, 2001). While satisfaction of a "'representative number' depends on whether one of skill in the art would recognize that Applicant was in possession of the necessary common attributes or features of the elements possessed by members of the genus in view of the species disclosed," the description of a single species may adequately support a genus (emphasis added, *Id.*).

As discussed above, the present specification discloses isolated nucleic acid having PAP sequences including those encoding SEQ ID NO:7 or having SEQ ID NO:2. In addition, the application discloses that the invention includes nucleic acids that hybridize to the complement

of those sequences under stringent hybridization conditions (page 17, lines 11-18), and nucleic acids that are 90% or more identical thereto. The claimed nucleic acids have particular structural and functional features, such as being 90% identical to a nucleotide sequence encoding SEQ ID NO:7 or the complement thereof, or capable of hybridizing to the complement of SEQ ID NO:2 or a nucleotide sequence encoding SEQ ID NO:7 under specific, disclosed conditions, which may encode a polypeptide that interacts with PBR (page 5, lines 19-24; page 34, lines 14-17), and so regulates progesterone synthesis (Example 5) or cholesterol transport (page 49, lines 19-22).

Thus, it is respectfully submitted that the claims recite functional characteristics coupled with a known or disclosed correlation between function and structure, and so are in compliance with the written description requirement of 35 U.S.C. § 112, first paragraph.

Accordingly, withdrawal of the § 112(1) rejections is appropriate and is respectfully requested.

Conclusion

Applicants respectfully submit that the claims are in condition for allowance, and notification to that effect is earnestly requested. The Examiner is invited to telephone Applicants' attorney at (612) 371-2106 to facilitate prosecution of this application.

If necessary, please charge any additional fees or credit overpayment to Deposit Account No. 19-0743.

Respectfully submitted,

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Patricia A Hoffman _____

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 25, Issue of September 5, pp. 15015-15022, 1990
Printed in U.S.A.

Peripheral-type Benzodiazepine Receptors Mediate Translocation of Cholesterol from Outer to Inner Mitochondrial Membranes in Adrenocortical Cells*

(Received for publication, February 27, 1990)

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In previous studies we demonstrated that peripheral-type benzodiazepine receptors (PBR) were coupled to steroidogenesis in several adrenocortical and Leydig cell systems (Mukhin, A. G., Papadopoulos, V., Costa, E., and Krueger, K. E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 9813–9816; Papadopoulos, V., Mukhin, A. G., Costa, E., and Krueger, K. E. (1990) *J. Biol. Chem.* 265, 3772–3779). The current study elucidates the specific step in the steroid biosynthetic pathway by which PBR mediate the stimulation in steroid hormone production. The adrenocorticotropin (ACTH)-responsive Y-1 mouse adrenocortical cell line was used to compare the mechanisms by which ACTH and PK 11195 (a PBR ligand) stimulate steroidogenesis. The effects of these agents were studied at three stages along the steroid biosynthetic pathway: 1) secretion of 20α-OH-progesterone by Y-1 cell cultures; 2) pregnenolone production by isolated mitochondrial fractions; 3) quantities of cholesterol resident in outer and inner mitochondrial membrane fractions. Steroid synthesis stimulated by ACTH was blocked by cycloheximide, an effect documented by other laboratories characterized by an accumulation of mitochondrial cholesterol specifically in the outer membrane. In contrast, PK 11195-stimulated steroidogenesis was not inhibited by cycloheximide, and the magnitude of the stimulation was markedly enhanced when the cells were pretreated with cycloheximide and ACTH. When isolated mitochondria were used, stimulation of pregnenolone production by PK 11195 was largely independent of exogenously supplied cholesterol, indicating that PBR act on cholesterol already situated within the mitochondrial membranes. This phenomenon was found to be the result of a translocation of cholesterol from outer to inner mitochondrial membranes induced by the PBR ligand. These studies therefore suggest that mitochondrial intermembrane cholesterol transport in steroidogenic cells is mediated by a mechanism coupled to PBR.

Extensive research has been devoted to delineating the steps underlying steroid hormone biosynthesis and its regulation. The primary point of control in the acute stimulation of steroidogenesis involves the first step in this biosynthetic pathway in which cholesterol is converted to pregnenolone by ($P\text{-}450_{scc}$),[1] an enzyme localized on inner mitochondrial membranes which is dependent on an electron transport system comprised of adrenodoxin and adrenodoxin reductase (1, 2). More detailed studies have shown that the reaction catalyzed by $P\text{-}450_{scc}$ is not rate limiting in the synthesis of steroid hormones, but rather it is the transport of the substrate cholesterol from intracellular stores to the inner mitochondrial membrane in which steroid production appears to be regulated (2–4).

Peptide hormones such as ACTH or gonadotropins act at their target tissues by binding to cell surface receptors to activate adenylate cyclase (5, 6) which then initiates a complex series of intracellular events ultimately increasing delivery of cholesterol to $P\text{-}450_{scc}$. Cholesterol is liberated from extramitochondrial stores (7, 8), transported to mitochondria (9, 10), incorporated into the outer mitochondrial membrane (11), and finally delivered to the inner mitochondrial membrane, where it is converted to pregnenolone by $P\text{-}450_{scc}$ (4, 12, 13).

Work by others has shown that the protein synthesis inhibitor cycloheximide blocks the stimulation by these hormones. This inhibition occurs at the step of intramitochondrial transport of cholesterol to the inner membrane (11). It is believed that a protein(s) with a rapid turnover rate is required to effect translocation within mitochondria and that the hormones may act either to increase the level of or to activate this protein(s) posttranslationally. Currently a number of proteins have been identified as potential candidates to participate in this scheme (14–16). Despite the fact that mitochondrial intermembrane cholesterol transport is paramount to understanding the regulation of steroidogenesis, this process, including identification of its functional components, has remained a mystery.

In what was previously a seemingly unrelated of area biochemistry, a drug binding site called the peripheral-type benzodiazepine receptor (PBR) was being studied by several laboratories. This drug receptor was originally discovered more than 10 years ago because it binds the benzodiazepine diazepam with relatively high affinity (17). The reason this drug receptor attracted much interest is that benzodiazepines are among the most highly prescribed drugs because of their pharmacological actions in relieving anxiety mediated through modulating the activity of γ-aminobutyric acid receptors in the central nervous system (18, 19). PBR were therefore discovered as another class of binding sites for benzodi-

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[1] The abbreviations used are: $P\text{-}450_{scc}$, C_{27} cholesterol side chain cleavage cytochrome P-450; ACTH, adrenocorticotropin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PBR, peripheral-type benzodiazepine receptor(s); 20-DHP, 20α-hydroxyprogesterone.

azepines distinct from the aforementioned neurotransmitter receptors. Although PBR were found in virtually all mammalian tissues, including the central nervous system, the function of this recognition site was not known despite the prevalent therapeutic use of benzodiazepines.

Further studies demonstrated that in addition to benzodiazepines, PBR bind other classes of organic compounds with high affinity (20–21). These studies led to the development of a photoaffinity probe that identified a protein of about 18 kDa (22). Subsequently our laboratory has purified this protein (23) and cloned, sequenced, and expressed the corresponding cDNA (24), demonstrating this protein to exhibit the binding properties of PBR. Other laboratories have identified another protein of about 30 kDa (25–27) which is likely to be associated with the 18-kDa protein; however, further clarification is required concerning its possible relationship with PBR.

In attempting to elucidate a function for PBR we considered two important findings. First, this receptor is found primarily on outer mitochondrial membranes (28, 29); and second, PBR are quite abundant in many tissues, but they are extremely abundant in steroidogenic cells (30–32). It appeared as if PBR were required to fulfill specialized physiological requirements of the host cell because of the great differences in mitochondrial density of PBR from different tissues and because there was no correlation between the density of PBR and the specific activities of some constitutive metabolic mitochondrial marker enzymes (32).

This information suggested that PBR are likely to play a role in steroidogenesis. Hence we have recently reported using a wide spectrum of ligands that bind to PBR and have demonstrated that these drugs, via binding to PBR, stimulate steroid biosynthesis in several adrenocortical (33) and Leydig cell systems (34). This effect was not blocked by cycloheximide and was also observed in *in vitro* reconstituted systems using isolated mitochondrial preparations. Furthermore, it was verified that PBR did not act by directly stimulating P-450$_{scc}$ (34).

The studies presented herein now provide evidence that PBR mediate translocation of cholesterol from the outer mitochondrial membrane to the inner membrane. These findings therefore imply that PBR are likely to comprise at least part of the mitochondrial intermembrane cholesterol translocation apparatus participating in the rate-determining step of steroid biosynthesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Measurement of Steroid Secretion—All details concerning propagation of Y-1 cells were described in detail elsewhere (33). For quantitation of steroid secreted Y-1 cells cultured in 12 × 22-mm wells were washed by three consecutive 30-min incubations with serum-free medium. The cells were then incubated in the presence of the indicated substances at a final volume of 1 ml of serum-free media at 37 °C. Measurement of 20-DHP in the media was performed by radioimmunoassay (33).

In many experiments, cells were subjected to a pretreatment as described here. Cultures were first washed by three changes of serum-free medium during 10-min intervals and then were incubated for 2 h at 37 °C in serum-free medium containing 0.2 mM cycloheximide and either 10^{-8} M ACTH or 1 mM dibutyryl adenosine 3′:5′-monophosphate. Results obtained with using ACTH *versus* the cAMP analogue were indistinguishable. Just prior to initiating time course studies, the cell cultures were rapidly washed three times with serum-free medium. For the remainder of the text this is referred to as pretreatment of the cells or preloading of the mitochondria.

Enzyme Assays—Measurement of activities for cytochrome *c* oxidase, 5′-nucleotidase, acid phosphatase, and NADPH-cytochrome *c* reductase, marker enzymes for inner mitochondrial membranes, plasma membranes, lysosomes, and endoplasmic reticulum, respectively, were performed as described in detail previously (32, 35). Type B monoamine oxidase was used as a marker for outer mitochondrial membranes and was determined with minor modifications of a published procedure (36). From 2 to 10 μg of protein was introduced into 200 μl of 10 mM sodium phosphate (pH 7.4), 10 μM [^{14}C]2-phenylethylamine (10 Ci/mol). After a 30-min incubation at 37 °C the reactions were stopped by adding 200 μl of 1 N HCl. Samples were extracted with 500 μl of 1:1 ethyl acetate:toluene (v/v), and radioactivity in the organic phases was measured. For all assays the enzymatic activities were verified to be proportional to the amount of protein used.

Isolation and Subfractionation of Mitochondria—Cells were scraped from culture dishes and homogenized in HS buffer (10 mM HEPES (pH 7.4), 320 mM sucrose) with a tight fitting Potter-Elvehjem tissue grinder. After the homogenate was centrifuged at 750 × *g* for 10 min the supernatant was recovered and centrifuged again at 750 × *g*. Following this, the supernatant was retrieved and centrifuged at 8000 × *g* for 10 min. The resulting membrane pellet was resuspended in the original volume of HS buffer and centrifuged again at 8000 × *g*, yielding an enriched mitochondrial preparation. Pregnenolone formation in mitochondrial fractions was determined as described before (34) in the presence of 0.1 μM trilostane to inhibit pregnenolone metabolism.

The final mitochondrial pellets accounted for 10–13% of the total cell protein. Relative to the initial cellular homogenates the following fold enrichment and total percent activity for each marker enzyme were recovered in these mitochondrial fractions: cytochrome *c* oxidase, 3.5-fold/42%; 5′-nucleotidase, 0.77-fold/8%; acid phosphatase, 1.8-fold/19%; NADPH-cytochrome *c* reductase, 0.66-fold/7%.

Subfractionation of mitochondrial membranes was performed according to the procedure of Schnaitman and Greenawalt (37) and optimized for Y-1 cell mitochondria according to the principle detailed by Dorbani *et al.* (38) to achieve maximum separation of outer and inner mitochondrial membranes. These modifications are detailed here as adapted for small scale studies. Mitochondrial fractions, corresponding to the equivalent of two Y-1 cell cultures in 175-cm^2 dishes, were suspended at a concentration of 20 μg of protein/μl in ice-cold HMS buffer (10 mM HEPES (pH 7.4), 230 mM mannitol, 70 mM sucrose) including 0.5% bovine serum albumin (w/v). An equal volume of HMS buffer containing 1.0% digitonin (w/v) was added to disrupt the outer mitochondrial membranes. After 15 min on ice, 6 volumes of HMS buffer with 0.5% bovine serum albumin was added, and the samples were triturated. Each sample was subjected to microcentrifugation (12,000 × *g*) in the cold for 5 min. The pellets were resuspended in 6 volumes of HMS buffer and centrifuged. The combined supernatants corresponded to the outer membrane fraction, but it also contained intermembrane space contents and a portion of the mitochondrial matrix. The pellets constituted the inner membrane fraction and also contained the remainder of the mitochondrial matrix as this represents partially disrupted mitoplasts. Greater than 80% of the marker enzymes for plasma membranes, lysosomes, and endoplasmic reticulum within these mitochondrial fractions was found to fractionate with the supernatant.

Cholesterol Translocation Assays—Mitochondrial fractions were suspended in 100 μl of HS buffer containing 0.76 mM aminoglutethimide, an inhibitor of P-450$_{scc}$, and incubated at 37 °C for 15 min. Translocation was initiated by adding 10 μl of 10 μM PK 11195 in HS buffer (also containing 0.1% ethanol). To control samples, 10 μl of HS buffer containing 0.1% ethanol was added. After 30 min at 37 °C translocation was terminated by the addition of 400 μl of ice-cold HS buffer. Following microcentrifugation for 10 min in the cold at 12,000 × *g* the mitochondrial pellet was subfractionated as described above.

Miscellaneous—Protein was measured by the method of Bradford (39) using ovalbumin with reduced mannose (Sigma) as a standard. Cholesterol was quantified by spectrofluorescence using a cholesterol oxidase assay (40). All data shown are representative of two to four independent experiments, each having replicate assays as indicated.

RESULTS

Relationship between ACTH- and PBR Ligand-stimulated Steroidogenesis—In our earlier studies (33, 34) we characterized PBR and the stimulation of steroidogenesis by PBR ligands in the mouse Y-1 (adrenal) and MA-10 (Leydig) cell lines. The properties of both cell lines were nearly identical in these aspects. MA-10 cells, however, exhibit an unusually strong response to its effector hormone, human choriogonadotropin, and therefore we have chosen to use the Y-1 cell

line as a more reliable model system for studying the acute stimulation of steroidogenesis.

The stimulation of steroidogenesis by human choriogonadotropin in MA-10 cells was found to be nonadditive with that of the stimulation by PBR ligands (34). To ensure that ACTH exhibited a similar relationship with PBR in Y-1 cells, the effect of 1 μM PK 11195 was determined with increasing concentrations of ACTH (Fig. 1). As reported earlier (33) PK 11195 produced about a 2-fold stimulation of 20-DHP synthesis in Y-1 cells. This stimulation was not additive with that by ACTH, as shown in Fig. 1. Rat and bovine adrenocortical cell preparations showed similar responses (Ref. 33 and data not shown), justifying the use of Y-1 cells as a model system for these studies. A clear interpretation of these results cannot be made at this point but might represent the possibility that physiological steroidogenic hormones may stimulate steroid biosynthesis by activation of some mechanism in which PBR participate (34).

At this point it should be indicated that experiments presented here only show results obtained with PK 11195. For all studies described here at least two other ligands exhibiting dissociation constants of less than 200 nM for PBR, signified as PK 14067 and Ro5-4864 in our earlier studies (33, 34), give results identical to those observed with PK 11195 reported herein whereas related ligands, clonazepam and flumazenil (with affinities poorer than 1 μM for PBR), show no effects.

Steroidogenesis Studied in Y-1 Cell Cultures—To begin elucidating the precise step in which PBR facilitate steroid synthesis, Y-1 cells were treated with ACTH or PK 11195 in the absence or presence of 0.2 mM cycloheximide. In the absence of the protein synthesis inhibitor ACTH produced a rapid stimulation in 20-DHP production which was sustained for over 3 h (Fig. 2*A*). PK 11195 also exhibited a rapid stimulation (within 10 min); however, after 30 min steroid formation had reached its completion. These kinetics were similar to those found previously in MA-10 cells (34).

In agreement with reports by other groups (4, 10, 11) ACTH no longer elicited a stimulation when Y-1 cells were simultaneously incubated with cycloheximide (Fig. 2*B*). In contrast, the stimulation by PK 11195 was unaffected by cycloheximide, a phenomenon that was also observed in MA-10 cells (34). This is presumably due to direct activation of PBR by the ligand.

At this point it was believed that the stimulation by PK 11195 was short lived because a specific steroidogenic pool of cholesterol in mitochondria may have been depleted. ACTH is known to mobilize cholesterol from extramitochondrial stores, and this might account for continued steroid production observed with this hormone. Cycloheximide was shown to block steroid production induced by ACTH at a step prior to conversion of cholesterol to pregnenolone (2, 3); however, loading of the outer mitochondrial membrane with cholesterol still occurs in the presence of this protein synthesis inhibitor (11). In agreement with this proposal, when cells were incubated with cycloheximide, ACTH, and PK 11195 simultaneously, progressive steroid synthesis was observed for at least 3 h (Fig. 2*B*, *solid triangles*) albeit the rate of synthesis was considerably lower than that observed with ACTH alone.

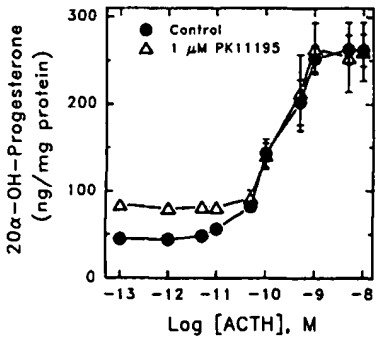

FIG. 1. **Effect of PK 11195 on ACTH-stimulated steroidogenesis.** Y-1 cells were incubated with the indicated concentrations of ACTH in the presence (*triangles*) or absence (*circles*) of 1 μM PK 11195. After 4 h 20-DHP secreted into the media was measured. Values are the means ± S.D. of triplicate assays.

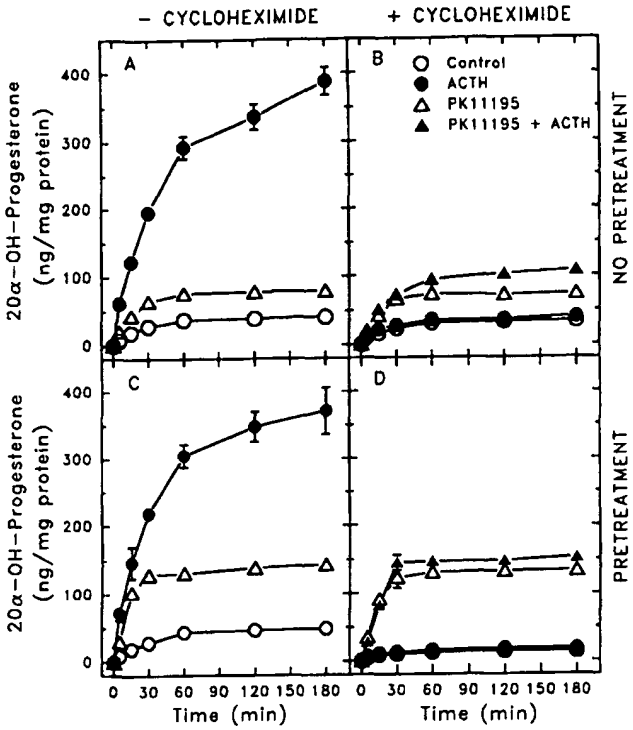

FIG. 2. **Comparison between ACTH- and PK 11195-stimulated steroidogenesis in Y-1 cell cultures.** Levels of 20-DHP production in Y-1 cells cultured without a pretreatment (*A* and *B*) or subjected to a pretreatment (*C* and *D*) performed by incubation for 2 h with 10^{-8} M ACTH and 0.2 mM cycloheximide are shown. Steroid synthesis was measured while in the absence (*A* and *C*) or presence (*B* and *D*) of 0.2 mM cycloheximide during an incubation with 10^{-8} M ACTH (*closed circles*), 10^{-6} M PK 11195 (*open triangles*), or no agents added to the medium (*open circles*). Also included in *B* and *D* are results in which PK 11195, ACTH, and cycloheximide were present simultaneously (*closed triangles*). Results are expressed as the means ± S.D. of three determinations.

Based on these findings we then determined whether pretreating Y-1 cells by preincubation for 2 h with cycloheximide and ACTH affected steroid synthesis regulated by PK 11195 (Fig. 2*C*). Using this pretreatment paradigm, the cells exhibited a slightly higher basal rate of steroid production as compared with control cells that were not pretreated. ACTH was still able to stimulate steroidogenesis despite the fact that the cells were exposed to cycloheximide for 2 h before. In pretreated cells, PK 11195 also induced a rapid stimulation of steroid synthesis; however, the pretreatment now permitted the ligand to be much more efficacious in stimulating steroid synthesis but with the same kinetics as observed with the ligand on control cells.

This scheme was also used to examine steroid synthesis in cells maintained in the presence of cycloheximide following the pretreatment period (Fig. 2*D*). Steroid production was blocked completely by cycloheximide whether the pretreated cells were coincubated with ACTH or no other components

were added to the culture medium. This finding corroborates the earlier observation that ACTH is incapable of stimulating steroidogenesis while in the presence of cycloheximide despite the fact that the mitochondria have already incorporated elevated levels of cholesterol available for steroidogenesis. In contrast, the stimulation by PK 11195 in pretreated cells was not affected by cycloheximide.

From the set of experiments given in Fig. 2 it can be hypothesized that ligand activation of PBR induces a rapid increase in steroid production, the magnitude of which is apparently related to cholesterol available for steroidogenesis within the outer mitochondrial membrane. Cholesterol incorporation into outer mitochondrial membranes by the preloading scheme seems to increase cholesterol levels for entry into this biosynthetic pathway. This permits activation of PBR to produce a greater level of steroidogenesis. Moreover, activation of PBR is insensitive to cycloheximide, implying that PBR function at or following the point in the biosynthetic pathway at which cycloheximide blocks ACTH-regulated steroidogenesis. If this hypothesis is correct one would expect similar results in mitochondria isolated from control and pretreated cells.

Pregnenolone Production in Mitochondrial Fractions—To support the proposal described in the preceding paragraph, control and pretreated cells were incubated with PK 11195 or ACTH in the presence of the P-450$_{scc}$ inhibitor aminoglutethimide for 1 h following which mitochondria were prepared from the cells (maintained in the presence of the inhibitor). Aminoglutethimide was washed from the mitochondrial fractions immediately prior to measuring the kinetics of pregnenolone formation (Fig. 3). Mitochondria from cells treated with ACTH showed a high rate of pregnenolone formation while coincubation of the cells with cycloheximide blocked this action (Fig. 3, *A* and *B*). Mitochondria from cells incubated with PK 11195 also showed an accelerated rate of pregnenolone formation which was not blocked by coincubation with cycloheximide. Similar results were found with mitochondria from pretreated cells except that PK 11195 produced a greater stimulation under these conditions (Fig. 3, *C* and *D*).

The findings with intact cells (Fig. 2) and the corresponding mitochondrial preparations (Fig. 3) are qualitatively equivalent, providing proof that within the cell, PBR mediate transport of cholesterol to the inner mitochondrial membrane which is utilized by P-450$_{scc}$ for the production of pregnenolone. Parallel experiments using [^{3}H]PK 11195 verified that >90% of the specifically bound PK 11195 had dissociated from the mitochondria during their isolation (data not shown). This indicates that the stimulatory action of PK 11195 was experienced before the mitochondria were isolated from the cells.

The experiments described thus far here or in our previous report (34) do not exclude the possibility that PBR are required for incorporation of extramitochondrial cholesterol into a pool available for steroidogenesis on the outer mitochondrial membrane. To demonstrate that the action of PBR is independent of extramitochondrial cholesterol the following experiment was performed. Mitochondria from control or pretreated cells were prepared in the presence of aminoglutethimide. After washing them free of the inhibitor, the mitochondria were suspended in buffer containing or lacking 100 μM cholesterol. PK 11195 was then introduced, and pregnenolone formation was measured (Fig. 4).

The PBR ligand stimulated pregnenolone formation from mitochondria of control or pretreated cells in a manner that was similar to 20-DHP production in whole cells, namely, mitochondria from pretreated cells exhibited a greater stim-

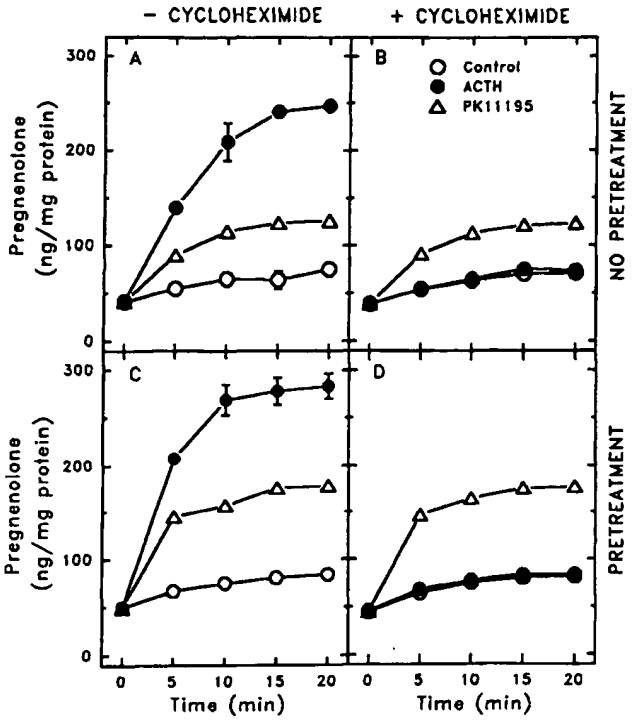

FIG. 3. **Pregnenolone production in mitochondria isolated from Y-1 cells treated with ACTH or PK 11195.** Control (*A* and *B*) or pretreated (*C* and *D*) Y-1 cells were incubated for 1 h in media containing (*B* and *D*) or lacking (*A* and *C*) 0.2 mM cycloheximide in addition to 0.76 mM aminoglutethimide, the P-450$_{scc}$ inhibitor. The following agents were included during this second incubation period: *open circles*, no agent; *closed circles*, 10^{-8} M ACTH; *triangles*, 10^{-6} M PK 11195. Mitochondria were then isolated in the continual presence of 0.76 mM aminoglutethimide. Following isolation the mitochondrial fractions were washed free of the inhibitor in HS buffer, and 15 mM sodium malate and 0.5 mM NADP were introduced following which pregnenolone was measured at the indicated times during incubation at 37 °C. Data are expressed as means and ranges of duplicate assays.

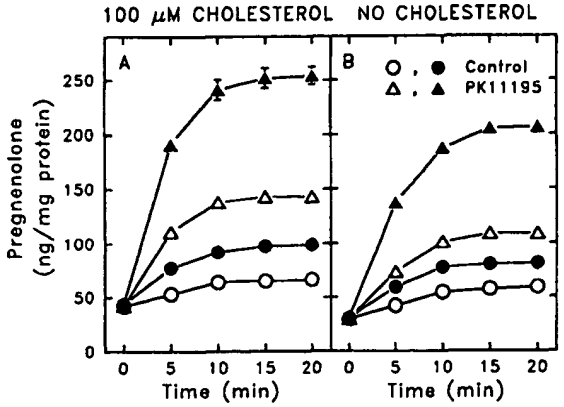

FIG. 4. **Increase of pregnenolone production in mitochondrial fractions treated with PK 11195.** Mitochondria were prepared from control (*open symbols*) or pretreated (*closed symbols*) cells and suspended in buffer containing (*A*) or lacking (*B*) 100 μM exogenous cholesterol with either 10^{-6} M PK 11195 (*triangles*) or no ligand (*circles*) and allowed to equilibrate at 37 °C for 5 min. At 0 min, 15 mM sodium malate and 0.5 mM NADP were introduced to the mitochondrial suspensions, and pregnenolone production was measured at the indicated times. Results are the means and ranges of duplicate determinations.

ulation by PK 11195. In the absence of exogenous cholesterol, the stimulation by PK 11195 was reduced by only 15%, demonstrating that PBR act on cholesterol already resident in the mitochondrial membranes. The slight reduction observed relative to when exogenous cholesterol is present could be accounted for by either a greater preservation of mitochondrial integrity in 100 μM cholesterol or such high concentrations of exogenous cholesterol may facilitate passive incorporation of cholesterol into the outer membrane. These experiments also provide further support that pretreatment of Y-1 cells results in an increase of mitochondrial cholesterol accessible for steroidogenesis which can be utilized by PBR to facilitate delivery of cholesterol to the P-450$_{scc}$-converting enzyme.

Translocation of Cholesterol from the Outer to the Inner Mitochondrial Membrane—Previous studies (2) have demonstrated that the rate-determining step in steroid biosynthesis occurs at the level of cholesterol transport to the inner mitochondrial membrane where P-450$_{scc}$ is localized. Because the previous experiments revealed that preloading the mitochondria magnifies the stimulation observed with PK 11195, it appeared as if ligand occupancy of PBR promoted cholesterol transport to P-450$_{scc}$.

To examine the likelihood that PK 11195 increases cholesterol transport to the inner mitochondrial membrane we initially examined a number of procedures to separate inner and outer mitochondrial membranes. Although other groups reported apparently efficient separation of these two membrane compartments by other methods in our hands Y-1 cell mitochondria could only be satisfactorily subfractionated using a method based on selective disruption of the outer mitochondrial membranes with digitonin (37, 38). This procedure was optimized to give the greatest degree of separation for cytochrome *c* oxidase and monoamine oxidase (Table I), marker enzymes for the inner and outer mitochondrial membranes, respectively. The supernatant fractions from this procedure contained about 85% of the monoamine oxidase activity and typically 60–70% of the total mitochondrial protein, consisting of outer membranes, intermembrane space, and a portion of the mitochondrial matrix. The remainder of the mitochondrial protein was in the pellet fraction also containing mitochondrial matrix and inner membranes characterized by ≥95% of the cytochrome *c* oxidase activity with the residual portion of monoamine oxidase. No significant differences were observed in comparing the subfractionation pattern of mitochondria from control or pretreated cells (Table I). Furthermore, as an important control for the following experiment, no differences in the subfractionation pattern were observed when the mitochondria were incubated with 0.76 mM aminoglutethimide and 1 μM PK 11195 for 30 min at 37 °C (data not shown).

To provide unequivocal evidence that PBR mediate translocation of cholesterol from the outer to the inner mitochondrial membrane, the following experiment was devised. Mitochondria from control and pretreated Y-1 cells were prepared in the presence of aminoglutethimide. PK 11195 was then introduced to these mitochondrial preparations (still in the presence of aminoglutethimide to prevent conversion of cholesterol to pregnenolone), and after 30 min at 37 °C cholesterol transport was stopped by rapid cooling to 4 °C. Inner and outer mitochondrial membranes were then separated, and cholesterol contents in both mitochondrial compartments were determined. Because all mitochondrial cholesterol is quantitatively associated with the inner and outer membranes (41), cholesterol quantitations of the pellet and supernatant fractions are indicative of cholesterol contents in the inner and outer mitochondrial membranes, respectively.

Outer mitochondrial membrane fractions from pretreated cells contained about 25% higher levels of cholesterol than the corresponding fractions from control cells (Fig. 5). This is in agreement with earlier findings by Privalle *et al.* (11) suggesting that ACTH promotes cholesterol accumulation in outer mitochondrial membranes when cycloheximide is present. This other study, however, reported a doubling of outer membrane cholesterol in rat adrenal mitochondria. The smaller increase observed here is evidently characteristic of Y-1 cells, which also exhibit a lower steroid output than primary adrenal cells.

Mitochondria from control cells exhibited a small increase in the content of cholesterol associated with the inner membrane fraction (Fig. 5, *left panel*). Introduction of PK 11195 doubled this increase, which corresponds to a 2-fold higher level of cholesterol in the inner membranes compared with that found initially. Since exogenous cholesterol was not supplied to the mitochondria, these increases in the inner membranes were accompanied by concomitant decreases of cholesterol in the outer membrane fractions.

Several notable differences were observed when mitochondria from pretreated cells were used (Fig. 5, *right panel*). Without addition of the PBR ligand a nearly 2-fold increase in inner membrane cholesterol was found, and this increase was still more pronounced with PK 11195. Pretreatment, therefore, is characterized by higher initial outer mitochondrial membrane levels, of which a substantially greater proportion is translocated to inner mitochondrial membranes. In this respect an unexpected finding was encountered with

TABLE I

Subfractionation of mitochondrial membranes

Mitochondria from control and pretreated Y-1 cells were subfractionated as described under "Experimental Procedures" after which cytochrome *c* oxidase and type B monoamine oxidase were measured. A representative experiment is shown in which recovery of total mitochondrial protein in the pellet and supernatant fractions was 41% and 59%, respectively. Results are expressed as means ± S.D. of four separate preparations.

Fraction	Cytochrome c oxidase			Monoamine oxidase		
	Specific activity	Enrichment	Recovery	Specific activity	Enrichment	Recovery
	nmol/min/mg protein	*-fold*	%	*μmol/min/mg protein*	*-fold*	%
Control						
Mitochondria	150 ± 14	1		1.39 ± 0.07	1	
Pellet	497 ± 27	3.31 ± 0.18	95 ± 1	0.65 ± 0.4	0.47 ± 0.03	14 ± 2
Supernatant	21 ± 3	0.13 ± 0.02	5 ± 1	2.47 ± 0.31	1.78 ± 0.22	86 ± 2
Pretreatment						
Mitochondria	119 ± 3	1		1.85 ± 0.02	1	
Pellet	476 ± 65	4.00 ± 0.55	96 ± 1	0.93 ± 0.15	0.50 ± 0.08	17 ± 3
Supernatant	16 ± 8	0.13 ± 0.07	4 ± 1	3.30 ± 0.32	1.78 ± 0.17	83 ± 3

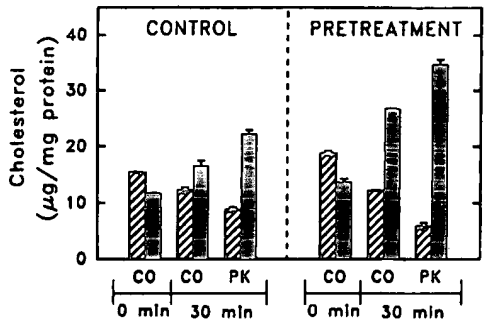

FIG. 5. **Translocation of cholesterol from outer to inner mitochondrial membranes.** Mitochondria from control or pretreated cells were prepared in HS buffer containing 0.76 mM aminoglutethimide (and lacking exogenous cholesterol). Translocation of cholesterol, as detailed under "Experimental Procedures," was measured in these mitochondrial fractions incubated in the presence (*PK*) or absence (*CO*) of 10^{-6} M PK 11195 for 30 min and compared with the cholesterol distribution before the incubation period (*CO, 0 min*). Cholesterol contents associated with outer membrane (supernatant) fractions (*hatched bars*) and inner membrane (pellet) fractions (*solid bars*) are shown as the means and ranges of two determinations from one representative experiment.

mitochondria of pretreated cells. Approximately two-thirds of the outer membrane cholesterol had been transferred to the inner membrane fraction upon addition of PK 11195. Such extreme alterations in cholesterol distribution were consistently observed in different translocation experiments. This may indicate that in addition to causing an accumulation of outer mitochondrial membrane cholesterol, pretreatment may effect another change in outer membrane cholesterol, making it more accessible for steroidogenesis and/or intramitochondrial transport.

These findings give direct proof for functional coupling between PBR and mitochondrial intermembrane cholesterol transport. The relative degrees of cholesterol redistribution in Fig. 5 are qualitatively similar to the levels of pregnenolone production observed previously in isolated mitochondria (Fig. 4*B*). It should be noted that the mitochondrial preparations under these conditions exhibited maximal pregnenolone formation of 200–300 ng/mg mitochondrial protein (Figs. 3 and 4) whereas the amount of cholesterol that is translocated (with PK 11195 on mitochondria from pretreated cells) corresponds to about 10,000 ng/mg mitochondrial protein. Other experiments have shown that this difference is lowered when isocitrate instead of malate is used to support $P\text{-}450_{scc}$ activity whereby a 3-fold elevation in the amount of pregnenolone is attained whether in the presence or absence of 1 μM PK 11195 (data not shown). This is supported further by the finding that when 1 mM NADPH and 1 mM NADP are added to the inner mitochondrial membrane fractions (following removal of aminoglutethimide) comparable levels of pregnenolone formation are achieved (370 ± 106 *versus* 927 ± 109 ng of pregnenolone/mg of protein when the mitochondria were previously in the absence or presence of 1 μM PK 11195, respectively). At this point the discrepancy in values between cholesterol transported compared with that utilized for steroidogenesis requires further investigation; these results may reveal a specific compartmentalization of steroidogenic cholesterol within the inner membranes or a partial disruption of some aspect of mitochondrial steroidogenic function resulting from subcellular fractionation.

DISCUSSION

Prior to the present studies it had been determined that the step at which the rate of steroid production is controlled is at the point of cholesterol transport to $P\text{-}450_{scc}$ located on the inner mitochondrial membrane (2, 4, 11). Despite the efforts of many laboratories over the last 2 decades, our understanding of intramitochondrial cholesterol transport to the first enzyme in the steroid biosynthetic pathway has progressed little because of an inability to identify molecular components participating in this process. The studies described in this report now present evidence that PBR are coupled to the transport of cholesterol from the outer to the inner mitochondrial membrane.

As was discussed in the introductory statement, it was merely the serendipitous property that PBR bind specific drugs which permitted the identification of this protein initially. The conspicuous characteristics of a mitochondrial localization and extreme abundance in steroidogenic cells led to the current study in which it has now been discovered that PBR play a role in mitochondrial cholesterol transport of steroidogenic cells.

There are three possibilities that can account for the facilitation of mitochondrial intermembrane cholesterol translocation by PBR ligands. 1) PBR may comprise all or part of the mitochondrial translocation apparatus. 2) PBR may function in an ancillary process that is required for translocation to occur. 3) PBR may inhibit the transport of cholesterol by associating with the translocator. For the first two cases, ligand binding by PBR could stimulate these respective activities directly. In the last situation binding of ligands to the PBR may relax the inhibitory restraint placed on the translocator.

When considering the feasibility of these possible roles the molecular nature of PBR should be taken into account. The protein that comprises the binding sites for PBR ligands has a very lipophilic amino acid composition (24), which is in accordance with what would be expected for a membrane cholesterol transporter or a modulator of the membrane-associated transporter. Two mechanisms proposed previously to account for mitochondrial intermembrane cholesterol transport include either 1) the cooperation of a carrier protein in the intermembrane space to effect delivery of cholesterol to the inner membrane (42), or 2) a direct transfer of cholesterol between membrane compartments at intermembrane contact points (43, 44). The present data do not favor either model, but the second possibility is attractive considering the hydropathic profile of the 18-kDa PBR protein. Despite being a relatively small protein it contains five potential transmembrane-spanning segments (24), denoting that it is not unreasonable to hypothesize that PBR may be embedded in both membrane bilayers at intermembrane contact points at which they might facilitate translocation of cholesterol between the outer and inner mitochondrial membranes.

Some evidence that supports this possibility is given in a recent report in which, in contradiction to several other studies, it was suggested that PBR are localized on inner mitochondrial membranes (45). This proposal requires further substantiation, especially when one considers that the 18-kDa PBR protein does not contain a leading mitochondrial signal sequence, a feature apparently common to all proteins that are translated in the cytoplasm and inserted into inner mitochondrial membranes (46). If PBR traverse both membranes, as was conceived above, this could help resolve discrepancies between different groups regarding the submitochondrial localization of PBR. Other outer mitochondrial membrane proteins residing at these contact points have been found apparently to cofractionate with the inner mitochondrial membrane (38).

As for the possibility in which PBR fulfill an ancillary

process required for cholesterol transport, this type of role is more difficult to envisage when considering the hydrophobic nature and unusually high abundance of PBR in steroidogenic mitochondria. If PBR carry out an auxiliary function necessary for translocation, this process might also be required by other mitochondrial systems. This possibility might explain why PBR are found in virtually all tissues.

An issue that requires special consideration is the relationship between the role of PBR and the mechanism of action of physiological activators of steroidogenesis such as ACTH. It was shown previously (7–11) that ACTH promotes cholesterol delivery from extramitochondrial locations for incorporation into outer mitochondrial membranes. Subsequent to this process cycloheximide inhibits the steroidogenic response of ACTH by inhibiting translocation of cholesterol to the inner mitochondrial membrane (11). The results shown here confirm these findings and demonstrate that PBR ligands overcome this block by cycloheximide, thereby reestablishing steroidogenesis. The pretreatment paradigm developed in these studies was found to increase the steroidogenic efficacy of PK 11195. Therefore, PBR evidently can utilize cholesterol incorporated into or acted upon in the outer mitochondrial membranes as a result of the hormone action.

At this point it cannot be determined whether PBR participate directly in mitochondrial cholesterol transport as triggered by ACTH. As indicated from another study (47), two separate processes for cholesterol translocation may exist: one that is utilized by ACTH, and the other that is coupled to PBR. This situation might be expected if PBR function in an ancillary role as discussed above. In retrospect, although the efficacy of ACTH is greater than that of PK 11195, this does not justify the existence of separate transport mechanisms. Different PBR ligands vary considerably in intrinsic steroidogenic efficacy, governed largely by their affinities for PBR (33, 34). Accordingly, it is plausible that ACTH may regulate PBR function in a manner that is only partially mimicked by PBR ligands. This difference may be accentuated by the possibility that ACTH can elicit multiple actions on mitochondria to regulate steroidogenesis.

Recently another group has reported an effect of GTP on the recruitment of cholesterol into the steroidogenic pathway (48). This phenomenon is very different from the role of PBR reported here, based on several criteria. The effect with PK 11195 is not diminished appreciably in the absence of exogenous cholesterol, nor is it affected by cycloheximide. Furthermore, GTP produces a stimulation greater than that observed with ACTH whereas PBR ligands show stimulations lower than and nonadditive to that of the hormone.

A final consideration that must still be given concerns this newfound function of PBR. These drug receptors are not just in steroidogenic cells but are apparently in most all mammalian cells, where in many cases an abundant density is detected (30, 32). Other questions arise when accounting for these observations. Are PBR required for transport of cholesterol in nonsteroidogenic mitochondria as well? Are PBR involved in the transport of other components to the inner mitochondrial membrane?

The translocation detailed here could be a mechanism that all mitochondria may use to incorporate cholesterol into their inner membranes. This possibility might help account for the effects of PBR ligands on mitochondrial respiration (21). To address the second question other studies have demonstrated that protoporphyrin IX can interact with PBR (25, 49). In a scheme analogous to steroid hormone synthesis, protoporphyrin IX is imported to the inner mitochondrial membrane where it is converted to heme. The transport apparatus for this system is yet to be identified. Although speculative at this point, these possibilities could be pointing to PBR or similar membrane components as playing central roles in mitochondrial function or membrane biogenesis.

Acknowledgments—We are indebted to Dr. Erminio Costa, of the Fidia-Georgetown Institute for the Neurosciences, whose keen insight was instrumental in initiating the studies examining the relationship between PBR and steroidogenesis. We are also grateful to Dr. Martin Dym, of the Dept. of Anatomy and Cell Biology, for his continued support and encouragement during the course of these studies.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 7, Issue of March 5, pp. 3772-3779, 1990
Printed in U.S.A.

The Peripheral-type Benzodiazepine Receptor Is Functionally Linked to Leydig Cell Steroidogenesis*

(Received for publication, September 21, 1989)

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Testicular mitochondria were previously shown to contain an abundance of peripheral-type benzodiazepine recognition site(s)/receptor(s) (PBR). We have previously purified, cloned, and expressed an M_r 18,000 PBR protein (Antkiewicz-Michaluk, Mukhin, A. G., Guidotti, A., and Krueger, K. E. (1988) *J. Biol. Chem.* 263, 17317–17321; (Sprengel, R., Werner, P., Seeburg, P. H., Mukhin, A. G., Santi, M. R., Grayson, D. R., Guidotti, A., and Krueger, K. E. (1989) *J. Biol. Chem.* 264, 20415–20421); and in this report, we present evidence that PBR are functionally linked to Leydig cell steroid biosynthesis. A spectrum of nine different ligands covering a range of over 4 orders of magnitude in their affinities for PBR were tested for their potencies to modulate steroidogenesis in the MA-10 mouse Leydig tumor cell line. The K_i for inhibition of [^{3}H]1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide binding and the EC_{50} for steroid biosynthesis for this series of compounds showed a correlation coefficient of $r = 0.95$. The most potent ligands stimulated steroid production by ~4-fold in these cells. This stimulation was not inhibited by cycloheximide, unlike human chorionic gonadotropin- or cyclic AMP-activated steroidogenesis. The action of PBR ligands was not additive to stimulation by human chorionic gonadotropin or cyclic AMP, but was additive to that of epidermal growth factor, another regulator of MA-10 Leydig cell steroidogenesis. Moreover, PBR ligands stimulated, in a dose-dependent manner, pregnenolone biosynthesis by isolated mitochondria when supplied with exogenous cholesterol. This effect was not observed with mitoplasts (mitochondria devoid of the outer membrane). Cytochrome P-450 side chain cleavage activity, as measured by metabolism of (22*R*)-hydroxycholesterol, was not affected by PBR ligands in intact cells. Similar results were also obtained with purified rat Leydig cells. In conclusion, PBR are implicated in the acute stimulation of Leydig cell steroidogenesis possibly by mediating the entry, distribution, and/or availability of cholesterol within mitochondria.

Benzodiazepines are one of the most widely used class of drugs in therapy due to their anxiolytic and anticonvulsant properties. It is now well established that besides their interaction with specific recognition sites located in the brain, benzodiazepines bind to membranes prepared from various tissues (1–4) containing the so-called peripheral-type benzodiazepine recognition site(s)/receptor(s) (PBR).[1] Tissue distribution of PBR showed their abundance in the adrenal gland and testis (3, 5, 6). Studies on the intracellular localization of PBR demonstrated that they are predominantly localized on the mitochondria and more precisely on the outer mitochondrial membrane (5–7).

We have recently purified, characterized, and cloned a protein comprising this receptor from rat adrenal gland mitochondria (8, 9). This protein has an M_r of about 18,000 and does not show significant sequence similarity to any other currently sequenced protein. Northern analysis has shown the presence of one RNA species of ~850 nucleotides exhibiting relative abundances qualitatively comparable with the densities of PBR in different tissues (9). Furthermore, expression of the corresponding cDNA in eukaryotic cells results in the expression of binding sites for PBR ligands (9).

Despite the accomplishments in elucidating the characteristics of this protein, the physiological role of this class of receptors has remained unknown, although they have been implicated in a number of cellular phenomena such as melanogenesis (10), hemoglobin synthesis (11), inhibition of cell proliferation (12), monocyte chemotaxis (13), protooncogene expression (14), and muscle contraction (15).

The possibility that PBR may play a role in the endocrine regulation of the adrenal gland and testis has first been raised by Anholt *et al.* (4), who showed that hypophysectomy induces a significant decrease in PBR density in both the adrenal gland and testis. Furthermore, it has been shown that different steroids are able to control the number of PBR in the rat testis (16) and that long-term administration of diazepam increases plasma testosterone levels in men (17), however, it does not have significant effects in rats (18). More direct evidence for a role of benzodiazepines in testicular function has been obtained by *in vitro* studies on decapsulated testes and interstitial cell suspensions where diazepam and Ro5-4864 have been shown to stimulate androgen production (18–20). But those studies did not demonstrate a direct action of the above-mentioned drugs on Leydig cells, the androgen-producing cell of the testis, or how these drugs act to stimulate

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[1] The abbreviations used are: PBR, peripheral-type benzodiazepine recognition site(s)/receptor(s); hCG, human chorionic gonadotropin; EGF, epidermal growth factor; Bt_2cAMP, dibutyryl adenosine 3′:5′-monophosphate; DBI, diazepam-binding inhibitor; P-450$_{scc}$, C_{27}-cholesterol side chain cleavage cytochrome P-450; Ro5-4864, 4′-chlorodiazepam; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide; PK 14105, 1-(2-fluoro-5-nitrophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide; PK 1406(7/8), (−/+)-*N*,*N*-diethyl-2-methyl-3-[4-(2-phenyl) quinolinyl]propranamide; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

androgen production. The use of decapsulated testes also limits the validity of these results, taking into account the regulation of Leydig cell steroidogenesis by paracrine factors of Sertoli cell origin (21, 22).

It is important to note that recently it has been reported that the endogenous DBI polypeptide, present in most central and peripheral tissues, is also expressed in testes (23, 24), and its presence has been primarily localized in Leydig cells.[2] DBI displaces PBR ligands from rat adrenal gland cortex (25, 26), which raises the possibility that DBI may interact physiologically with PBR and elicit an as yet undefined biological activity. Recently, another laboratory (27, 28) has reported the purification of an 8.2-kDa polypeptide from bovine adrenal fasciculata which exhibited the ability to stimulate pregnenolone synthesis in mitochondrial preparations of adrenal glands. This polypeptide was subsequently sequenced and determined to be DBI (29). These findings support the possibility that PBR and DBI may both cooperate in the process of steroid biosynthesis.

The biosynthesis of steroid hormones begins with the transport of the substrate cholesterol from extramitochondrial stores to the first enzyme in the pathway, cytochrome P-450$_{scc}$, located on the inner mitochondrial membrane. This is the rate-limiting step of steroidogenesis and the main site for regulation by physiological stimuli during acute stimulation (30, 31). The localization of PBR on the mitochondrial compartment, the above-mentioned preliminary studies, and the finding that DBI is localized in Leydig cells raised the possibility that PBR may participate in the regulation of testicular steroidogenesis and prompted us to examine whether PBR ligands could alter Leydig cell function. For this purpose, two cell models were used: the mouse Leydig cell line MA-10 and purified rat Leydig cells.

EXPERIMENTAL PROCEDURES

Cells—The MA-10 cell line originally cloned from the solid M5480P mouse Leydig cell tumor (32) used in these experiments was generously given by Dr. Mario Ascoli (The Population Council, Rockefeller University, New York). Stock cultures were grown in modified Waymouth's MB752/1 medium containing 20 mM HEPES, 1.2 g/liter $NaHCO_3$, and 15% horse serum, pH 7.4, as described by Ascoli (32). Before use, the MA-10 Leydig cells grown in 12 × 22-mm wells were washed three times, at 30-min intervals, with 1 ml of serum-free media to eliminate serum components that may interfere with the assays and were incubated for the indicated periods of time in the presence of the indicated substances. Testicular interstitial cells were prepared by collagenase dissociation (33) of testes obtained from adult Sprague-Dawley rats (300 g). This preparation contained 20–30% 3β-hydroxysteroid dehydrogenase-positive cells (Leydig cells). Leydig cells were further purified using discontinuous Percoll gradient centrifugation as previously described (33). The preparations obtained contained 75–85% Leydig cells as shown by histochemical staining for 3β-hydroxysteroid dehydrogenase (33).

[^{3}H]PK 11195 Binding Assays and Photolabeling of PBR—Cells were scraped from 75-cm^2 culture flasks into 5 ml of Kreb's buffer (154 mM NaCl, 60 mM KCl, 3 mM $CaCl_2$, 1.5 mM KH_2PO_4, 1.5 mM $MgSO_4$, 20 mM Na_2PO_4, pH 7.4), dispersed by trituration, and centrifuged at 1200 × *g* for 5 min. The cell pellets were resuspended in buffer, and larger cell aggregates were allowed to settle to the bottom of the tube before the cell suspension was retrieved for experimentation.

[^{3}H]PK 11195 binding studies on 5 μg of protein from the cell suspensions were performed in 250–400 μl of Kreb's buffer at 37 °C, essentially the same conditions under which the effects of PBR ligands on steroidogenesis were studied. Nonspecific binding was determined in the presence of 10 μM PK 11195. After 30 min, the assays were stopped by filtration through Whatman GF/C filters and washed with 20 ml of 25 mM Tris-HCl, pH 7.4, containing 1 μM PK 11195. Radioactivity trapped on the filters was determined by liquid scintillation counting. Total binding accounted for <10% of the radioligand introduced, whereas specific binding was >85% of the total binding at all radioligand concentrations used.

Photolabeling of MA-10 cell mitochondria was performed by suspending 2 mg of membrane protein in 2 ml of 25 mM Tris-HCl, pH 7.4, 0.32 M sucrose buffer which contained 9 nM [^{3}H]PK 14105. The membranes were preincubated at 4 °C for 30 min before being placed in a rectangular dish (75 × 52 × 5 mm), continuously rocked for 60 min at 4 °C, and simultaneously irradiated from a distance of 2 cm using an UVGL-58 ultraviolet light (UVP, Inc., San Gabriel, CA) with maximum emission at 366 nm. Following irradiation, 10 μl of 2 mM PK 11195 was added to the membrane suspensions. The samples were incubated for another 30 min, diluted into 10 ml of Tris/sucrose buffer, and centrifuged at 20,000 × *g* for 10 min; and the pellets were washed once more in 10 ml of buffer.

Steroid Biosynthesis—These experiments were performed with the MA-10 Leydig cells plated in 12 × 22-mm wells and incubated for the times shown in the presence of the indicated substances at a final volume of 1 ml of serum-free media at 37 °C. Where purified rat Leydig cells were used, 50,000 cells/500 μl of serum-free media were incubated in borosilicate culture tubes at 32 °C. At the end of the incubation period, the cell media were saved, centrifuged at 1,500 × *g* for 10 min, and stored at −20 °C until used. Cells were dissolved with 0.1 N NaOH for protein measurement.

Progesterone and 20α-hydroxyprogesterone production for the MA-10 cells and testosterone accumulation for the rat Leydig cells were measured by means of radioimmunoassay. Antibody to progesterone was obtained from Endocrine Sciences (Tarzana, CA), and the assay was performed as described by the manufacturer. Antibody to 20α-hydroxyprogesterone was a gift from Dr. G. Nieswender (Colorado State University), and the assay was performed as described (34). Anti-testosterone antibody was obtained from ICN Co. (Lisle, IL), and the assay was performed as described by the manufacturer. Analysis of the radioimmunoassay data was performed using the IBM-PC RIA Data Reduction program (version 4.1) obtained from Jaffe and Associates (Silver Spring, MD).

Measurement of Cholesterol Transport in Mitochondria—MA-10 Leydig cells were washed with serum-free media as described above followed by washing in phosphate-buffered saline. Mitochondria were then prepared as previously described (35, 36). The effect of different compounds on cholesterol transport and/or on P-450$_{scc}$ activity was determined as previously described (28, 35) by incubating mitochondria with the ligands in the presence of exogenous cholesterol (100 μM) and trilostane (0.1 μM), an inhibitor of pregnenolone metabolism (37). The production of pregnenolone was measured after a 10-min incubation with a specific radioimmunoassay (38) using an antibody given by Dr. F. F. G. Rommerts (Erasmus University, Rotterdam, Netherlands). In another series of experiments, MA-10 Leydig cell mitoplasts were prepared by the method of Schnaitman and Greenawalt (Ref. 39; see also Ref. 35) and incubated under the same conditions described above for mitochondria. The purity of mitoplasts was examined by measuring cytochrome-*c* oxidase and Amytal-insensitive NADH:cytochrome-*c* reductase as markers for inner and outer mitochondrial membranes, respectively (36). Values for these enzymes in mitoplasts were 4.2 ± 0.5 mol/min/mg of protein and 38 ± 2.3 nmol/min/mg of protein corresponding to a 3-fold increase and a 10-fold decrease, respectively, of the specific activity for each enzyme compared to whole mitochondria.

Protein Measurement—Protein was measured by the method of Bradford (40) using bovine serum albumin as a standard.

Materials—Purified human chorionic gonadotropin (hCG; batch CR-125 with a biological potency of 11,900 IU/mg) was a gift from the National Institutes of Health. Purified mouse epidermal growth factor (EGF) was purchased from Sigma. [1,2,6,7-^{3}H]Progesterone (specific activity of 94.1 Ci/mmol), 20α-[1,2-^{3}H]hydroxyprogesterone (specific activity of 45 Ci/mmol), [1,2,6,7-^{3}H] testosterone (specific activity of 93.9 Ci/mmol), [7-^{3}H pregnenolone (specific activity of 22.6 Ci/mmol), and N-[*methyl*-^{3}H]PK 11195 were obtained from Du Pont-New England Nuclear. [^{3}H]PK 14105 was purchased from Research Products International Corp. PK 11195, PK 14067, and PK 14068 were the gift of Dr. C. Gueremy (Pharmuka Laboratoire Groupe, Rhône-Poulenc Sante, Gennevilliers, France). Zolpidem was generously donated by Dr. S. Z. Langer (Synthelabo Recherche, Paris, France). Diazepam, clonazepam, flumazenil, and Ro5-4864 were a gift from Hoffmann-La Roche. Aminoglutethimide was provided by Ciba-Geigy, and trilostane was from Sterling-Winthrop (New York, NY). Percoll was purchased from Pharmacia LKB Biotechnology Inc. All

[2] P. Bovolin, J. Miyata, J. Schlichting, C. Ferrarese, A. Guidotti, and H. Alho, *Regul. Pept.*, submitted for publication.

cell culture supplies were purchased from GIBCO, and cell culture plasticware was from Corning. All other chemicals were of analytical quality and were obtained from commercial sources.

RESULTS

Characterization of PBR in MA-10 Cells—Kinetics of [^{3}H] PK 11195 binding at 37 °C to MA-10 cells revealed that specific binding of this ligand was rapid, reaching steady-state conditions after 10 min (data not shown). Furthermore, if 10 μM PK 11195 was subsequently added, specific radioligand binding was completely displaced within 15–20 min. Scatchard analysis demonstrated a single class of binding sites with a dissociation constant of 1.8 nM at a density of 56 pmol/mg of protein (Fig. 1). Photolabeling of MA-10 cell mitochondrial fractions with [^{3}H]PK 14105 verifies that these cells contain the 18-kDa protein which constitutes the binding domains for PBR ligands (9).

Specificity of these binding sites was determined using nine different ligands from several classes of organic compounds known to interact with PBR. These ligands exhibited a rank order potency to compete against [^{3}H]PK 11195 binding which is consonant with their relative potencies reported by other groups (Fig. 2). The most notable observations are that the isoquinoline carboxamide PK 11195 was the most potent compound of this series. The quinoline propranamide PK 14067 was about 200-fold more potent than its enantiomer, PK 14068 (41). Of the benzodiazepines, Ro5-4864 was the most potent derivative, whereas clonazepam and flumazenil, an antagonist of central benzodiazepine actions, exhibited low affinities for PBR.

Effect of PBR Ligands on MA-10 Leydig Cell Steroid Biosynthesis—To investigate whether the different ligands affected Leydig cell steroidogenesis, increasing concentrations of these compounds were incubated for 4 h with MA-10 cells, and their effects on progesterone production were measured (Fig. 3). The three most potent compounds of this series were very efficacious at stimulating progesterone synthesis, whereas the less potent compounds either achieved a lower level of maximal stimulation or did not have an effect on steroid production. PK 11195 and PK 14067 were the most potent and produced a 3–4-fold stimulation of progesterone production. Among the benzodiazepines used, Ro5-4864 was

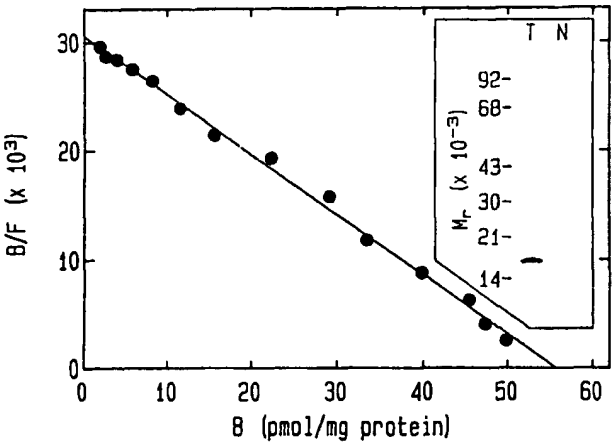

FIG. 1. **Scatchard analysis of [^{3}H]PK 11195 binding in MA-10 Leydig cells.** Specific binding was measured using radioligand in a concentration range of 0.07–20 nM. *Inset*, MA-10 cell mitochondrial fractions photolabeled with [^{3}H]PK 14105 in the absence (*lane T*) or presence (*lane* N) of 10 μM PK 11195, which were subjected to electrophoresis, transferred to nitrocellulose, and autoradiographed as described (8).

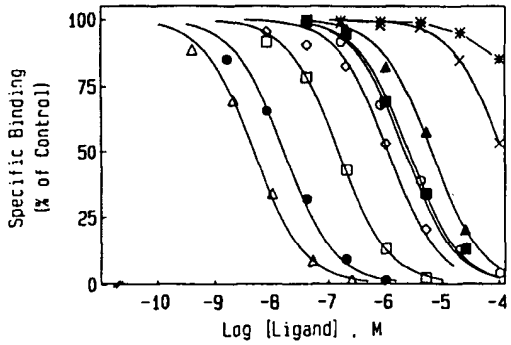

FIG. 2. **Binding specificity of PBR.** Specific binding of [^{3}H]PK 11195 (0.8 nM) to MA-10 cells was measured in the presence of the indicated concentrations of each competing ligand. Curves were generated by computer-assisted nonlinear regression analysis according to a least-squares curve-fitting program. △, PK 11195; □, Ro5-4864; ■, diazepam; ▲, flunitrazepam; ×, clonazepam; ●, PK 14067; ○, PK 14068; ◇, zolpidem; *, flumazenil.

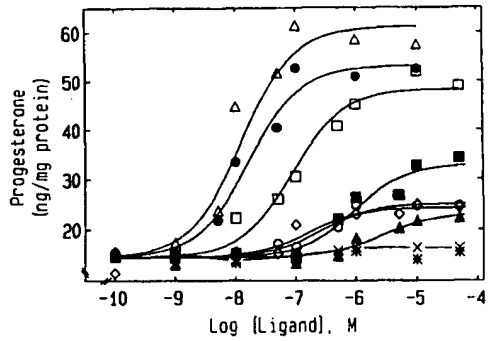

FIG. 3. **Stimulation of steroidogenesis by PBR ligands in MA-10 cells.** Cultures were incubated for 4 h in media containing the indicated concentrations of each ligand. Progesterone was measured as described in the text. Curve fitting was performed essentially as described for Fig. 2 except that the maximal stimulation was held variable so as to estimate this parameter for each ligand. Symbols are listed in the legend to Fig. 2. Values are means of two to four experiments conducted in triplicate. Standard deviations are not given for the simplicity of the figure and were <10% of the mean values.

the most potent, giving a 3.5-fold increase in steroidogenesis, whereas diazepam attained only a 2-fold maximal stimulation. The other compounds used significantly stimulated steroid production (1.5-fold) only at concentrations higher than 10^{-6} M, whereas clonazepam and flumazenil were completely inactive at concentrations above 10^{-5} M.

When comparing the EC_{50} values of the stimulatory effect of the different PBR ligands with the inhibitory constants with which these ligands compete for [^{3}H]PK 11195 binding to the MA-10 cells (Fig. 4), an excellent correlation was observed ($r = 0.95$). This provides strong evidence that the effects of these drugs on steroidogenesis are consequent to their binding to PBR. PK 11195 (10^{-6} M) and Ro5-4864 (10^{-5} M) also stimulated 20α-hydroxyprogesterone production by MA-10 Leydig cells to similar extents as observed with progesterone (data not shown), indicating that their actions are not unique to progesterone and that there is an increase of all steroidogenic products of these cells. This implies an action at the level of the initial substrate (cholesterol) and its processing. It is important to note at this point that all compounds used in these studies did not cross-react with the antibodies used in the different radioimmunoassays.

Effect of PBR Ligands on hCG- and EGF-stimulated Ste-

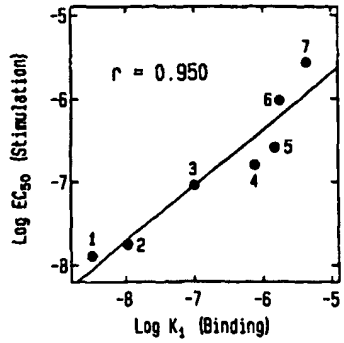

FIG. 4. **Correlation between binding affinities and stimulatory potencies of PBR ligands.** The K_i estimated for each compound from the analysis of Fig. 2 is compared to the concentration of ligand required to exhibit one-half of the respective maximal stimulation as was determined in Fig. 3. *Point 1*, PK 11195; *point 2*, PK 14067; *point 3*, Ro5-4864; *point 4*, zolpidem; *point 5*, PK 14068; *point 6*, diazepam; *point 7*, flunitrazepam.

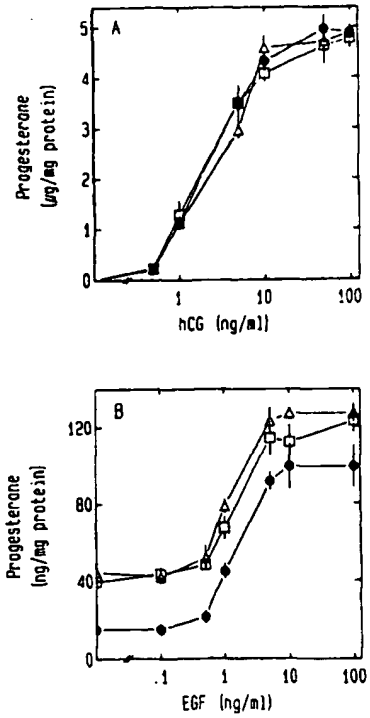

FIG. 5. **Effects of PBR ligands on hCG- and EGF-stimulated steroidogenesis.** MA-10 Leydig cells were incubated for 4 h in the presence of 10^{-6} M PK 11195 (Δ) or 10^{-5} M Ro5-4864 (□) with increasing concentrations of hCG (*A*, ●) or EGF (*B*, ●). Cell media were collected, and progesterone accumulation was measured by radioimmunoassay. Values are means ± S.D. of two independent experiments (n = 4). Note the different units used on the *abscissas* for both panels.

roidogenesis—MA-10 Leydig cells respond to hCG in a dose-dependent manner, with a half-maximal stimulation at 3–5 ng/ml (Fig. 5*A*). Maximal stimulation of progesterone production (330-fold) was obtained with 25 ng/ml hCG. Addition of 10^{-6} M PK 11195 or 10^{-5} M Ro5-4864 to increasing amounts of hCG did not affect the responses obtained by hCG alone (Fig. 5*A*), indicating that PBR ligands and hCG may act through a common pathway. To further examine whether PBR ligands may modulate the Leydig cell response to this hormone, 0.5 ng/ml hCG was used in the presence and absence of increasing amounts of PK 11195. In two separate experiments, we were unable to show any modification of hCG (0.5 ng/ml)-stimulated progesterone production by MA-10 cells at concentrations of PK 11195 up to 10^{-6} M (206 ± 17.8 ng of progesterone/mg of protein without PK 11195 *versus* 221 ± 19.7 ng of progesterone/mg of protein with 10^{-6} M PK 11195).

Since hCG is an unusually potent activator of steroidogenesis in MA-10 Leydig cells in comparison with normal Leydig cells, we also examined the effect of PBR ligands on the responses of Leydig cells to EGF, another modulator of their function. In Fig. 5*B*, a dose-response curve of EGF on Leydig cell progesterone production is shown. Maximal stimulation of steroidogenesis (6-fold) was obtained with 10 ng/ml EGF. PK 11195 and Ro5-4864 increased Leydig cell steroid output in the presence of all concentrations of EGF used, indicating that EGF and PBR ligands stimulate steroidogenesis in MA-10 Leydig cells by two different mechanisms.

Time course studies on the effect of the steroidogenic active PBR ligands on Leydig cell progesterone production were undertaken and compared with the results obtained from time course studies using hCG and EGF (Fig. 6). As early as 10 min following exposure to the cells, PK 11195 and Ro5-4864 stimulated steroid biosynthesis above the basal rate of synthesis; and after 40 min progesterone synthesis had reached its completion. In contrast, the stimulation observed with EGF exhibited a slower response, and maximal steroid accumulation was achieved after 3 h. It is important to point out that hCG-stimulated progesterone production was also observed within 10 min, as was seen with the PBR ligands, but it reached a plateau after 4 h (Fig. 6). These findings further support the implication that PBR ligands act through a mechanism different from that employed by EGF but which

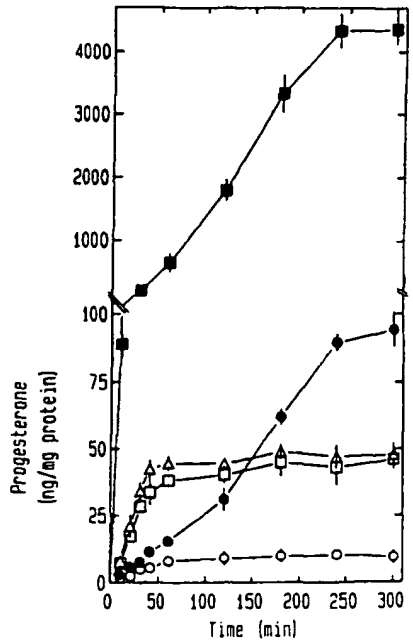

FIG. 6. **Time course on effects of PBR ligands, hCG, and EGF on MA-10 steroid biosynthesis.** MA-10 Leydig cells were incubated for the times shown in the absence (○) or presence of 10^{-6} M PK 11195 (Δ), 10^{-5} M Ro5-4864 (□), 10 ng/ml EGF (●), or 50 ng/ml hCG (■). At the end of the incubation period, media were collected, and progesterone was measured by radioimmunoassay. Values are means ± S.D. from two independent experiments (n = 4) plotted on two separate scales. In parallel experiments, incubation of the cells with 1 mM Bt_2cAMP gave results which were equivalent to those with hCG.

may be included in the multiple cellular events affected by hCG.

Studies on Mechanism of Action of PBR Ligands on Leydig Cell Steroidogenesis—In search for the site of action of PBR ligands on steroid biosynthesis, we first examined the possibility that PBR ligands may affect protein synthesis, which in turn will affect steroidogenesis. In the presence of the protein synthesis inhibitor cycloheximide (0.2 mM), hCG or Bt_2cAMP-stimulated steroidogenesis is almost completely inhibited, although a slight but significant stimulation remains (Table I). In contrast, progesterone production stimulated by 10^{-6} M PK 11195 or 10^{-5} M Ro5-4864 was not inhibited by cycloheximide. At submaximal concentrations of hCG (0.5 ng/ml), steroid production was still greater than that observed with the PBR ligands; cycloheximide, however, completely inhibited steroid production at these hCG concentrations (Table I). These findings demonstrate that cycloheximide does not inhibit the stimulation by PBR ligands, unlike the stimulation observed with hCG.

Table I also shows that stimulation of steroid biosynthesis by hCG, Bt_2cAMP, or PBR ligands is inhibited by the P-450_{scc} inhibitor aminoglutethimide (42, 43). Furthermore, progesterone production under conditions of unlimited substrate, as in the presence of exogenous pregnenolone, was not affected by hCG, Bt_2cAMP, or PBR ligands (data not shown), indicating that their acute regulatory effects on steroidogenesis are prior to pregnenolone metabolism.

At this point, we also examined the possibility that ligand binding to PBR may affect cholesterol metabolism to pregnenolone by directly acting on the mitochondrial P-450_{scc} enzyme. The metabolism of (22*R*)-hydroxycholesterol to progesterone by MA-10 cells was used as an index for P-450_{scc} activity (44). Ro5-4864 (10^{-5} M) did not affect progesterone production in the presence of 0.25 μM (22*R*)-hydroxycholesterol (244.67 ± 42.52 *versus* 239.67 ± 30.33 ng of progesterone/mg of protein/4 h; means ± S.D., $n = 3$) or in the presence of 100 μM (22*R*)-hydroxycholesterol (maximal concentration) (5047.00 ± 238.04 *versus* 4977.33 ± 213.30 ng of progesterone/mg of protein/4 h; means ± S.D., $n = 3$). Similar results were observed using other (22*R*)-hydroxycholesterol concentrations (data not shown), showing that the mechanism of stimulation by PBR is not by direct activation of P-450_{scc}. These findings and the fact that PBR are essentially located on the outer mitochondrial membrane prompted us to examine whether this receptor plays a role in cholesterol transport, the rate-limiting step of steroidogenesis.

To investigate the effect of PBR in cholesterol transport *in vitro*, PBR ligands were added to Leydig cell mitochondria incubated in the presence of exogenous cholesterol and trilostane, an inhibitor of 3β-hydroxysteroid dehydrogenase. Fig. 7*A* shows the results obtained from these experiments. PK 11195 and Ro5-4864, in the same concentration ranges that increased intact MA-10 cell steroidogenesis, stimulated pregnenolone production in Leydig cell mitochondria, whereas 10^{-5} M clonazepam was without any effect. To ensure that these effects were mediated through the outer mitochondrial membrane (the site where PBR are localized), similar experiments were performed with mitoplasts (mitochondrial preparations from which the outer mitochondrial membrane was removed). Mitoplasts therefore contain the inner mitochondrial membrane with its constituent P-450_{scc} activity. The results obtained (Fig. 7*B*) showed that PBR ligands did not have an effect on pregnenolone production from mitoplasts, further demonstrating that they do not act directly on the P-450_{scc} enzyme. Therefore, from these findings, it can be deduced that PBR are most likely involved with cholesterol uptake from intracellular stores into mitochondria and/or promoting cholesterol availability to P-450_{scc}.

Regulation of Steroidogenesis by PBR Ligands in Leydig Cells Purified from Rat Testis—To exclude the possibility that the involvement of PBR in steroidogenesis may be specific to the MA-10 cell line rather than representative of normal Leydig cells, we also examined the action of PBR ligands on purified rat Leydig cell steroidogenesis. Table II shows that

TABLE I

Effects of cycloheximide and aminoglutethimide on MA-10 Leydig cell steroidogenesis

MA-10 Leydig cells were incubated for 4 h with or without cycloheximide or aminoglutethimide and the substances indicated. Progesterone production was measured by radioimmunoassay in cell media. Results are the means ± S.D. from two experiments ($n = 4$).

Treatments	Progesterone		
	No additions	Cycloheximide (0.2 mM)	Aminoglutethimide (0.76 mM)
	ng/mg protein/4 h		
Control	13.25 ± 2.50	12.25 ± 2.87	13.50 ± 2.64
hCG (50 ng/ml)	4824 ± 131	33.25 ± 13.12	14.50 ± 3.69
Bt_2cAMP (1 mM)	4772 ± 245	37.00 ± 10.86	13.75 ± 2.21
hCG (0.5 ng/ml)	201 ± 16	13.40 ± 2.56	14.10 ± 2.83
PK 11195 (10^{-6} M)	49.00 ± 6.27	47.00 ± 7.61	16.00 ± 0.81
Ro5-4864 (10^{-5} M)	45.50 ± 4.43	45.50 ± 6.02	15.50 ± 1.73

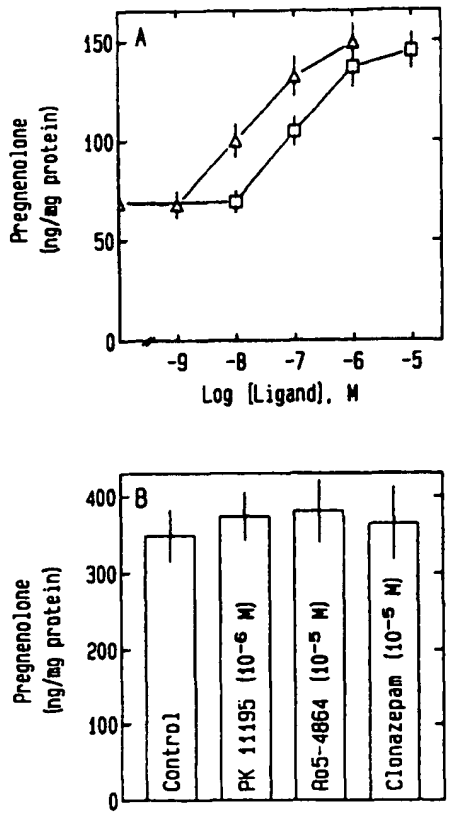

FIG. 7. **Effect of PBR ligands on pregnenolone formation by MA-10 Leydig cell mitochondria and mitoplasts.** Mitochondria (*A*) or mitoplasts (*B*) were incubated with cholesterol (100 μM), trilostane (0.1 μM), and the indicated concentrations of PK 11195 (△) or Ro5-4864 (□) or clonazepam. Incubations were performed for 10 min at 37 °C, and pregnenolone was then measured by radioimmunoassay. Note that in *A* the values for clonazepam are not shown since they are equal to those obtained in the absence of ligands. Results of one representative experiment are shown. Values are means ± S.D. derived from triplicate determinations.

TABLE II

Effects of PBR ligands on basal and hCG-stimulated rat Leydig cell steroidogenesis

Purified rat Leydig cells were incubated with the indicated additions for 4 h at 32 °C. Testosterone was measured by radioimmunoassay. Results of a representative experiment are shown as conducted in triplicate assays. Means ± S.D. ($n = 3$) are given.

Treatment	Testosterone	
	No additions	hCG (50 ng/ml)
	ng/10⁵ cells/4 h	
Control	1.30 ± 0.20	15.13 ± 2.05
PK 11195 (10^{-6} M)	2.33 ± 0.21	14.86 ± 1.51
PK 14067 (10^{-6} M)	2.20 ± 0.30	14.13 ± 1.49
PK 14068 (10^{-5} M)	1.16 ± 0.15	13.50 ± 1.51
Ro5-4864 (10^{-5} M)	2.40 ± 0.30	14.10 ± 2.26
Diazepam (10^{-5} M)	1.90 ± 0.10	14.96 ± 3.09
Clonazepam (10^{-5} M)	1.16 ± 0.11	14.90 ± 2.22

PK 11195 and Ro5-4864 stimulate, by about 2-fold, testosterone production by purified rat Leydig cells, whereas diazepam was less potent, and clonazepam had no effect. Moreover, stimulation of rat Leydig cell steroidogenesis was stereoselective for PBR, as demonstrated using the optical isomers PK 14067 and PK 14068. The hCG-stimulated testosterone production was not affected by PBR ligands (Table II). The site of PBR ligand action in rat Leydig cell steroid biosynthesis was also located, as was performed in MA-10 cells, at the level of cholesterol transport into mitochondria, the rate-limiting step of steroidogenesis (data not shown). These results verify that the effects on steroidogenesis mediated by PBR are also found in normal rat Leydig cell preparations. Hence, the MA-10 cell line has proven to be a suitable model system to examine the role of PBR in Leydig cell function.

DISCUSSION

The experiments reported here were designed to investigate the role of PBR in Leydig cell function. It has been previously shown (3, 5–7) that PBR are abundant in steroidogenic tissues and are predominantly localized on the outer mitochondrial membrane. A number of preliminary reports (18–20, 29, 45) show that some PBR ligands can increase steroidogenesis; however, a detailed pharmacological analysis was lacking to establish whether the mediator of this action was PBR. This study establishes that PBR play an important role in steroid biosynthesis and that their subcellular localization is consistent with current knowledge on the mitochondrial compartments participating in steroidogenesis.

In vitro studies using decapsulated testes or interstitial cell suspensions have shown that benzodiazepines (diazepam and Ro5-4864) stimulate androgen production (18–20). In view of the complexity of testicular structure and the role of cell-cell interactions in the testis (21, 22), we decided to examine the participation of PBR in Leydig cell steroidogenesis. For this purpose, two model systems, the mouse tumor MA-10 cell line and Leydig cells purified from rat testis, were used to demonstrate an excellent correlation between the affinities of nine ligands for PBR and their potencies to stimulate steroidogenesis.

MA-10 cells were found to contain a density of PBR which corresponds to ~6×10^7 binding sites/cell, representing about 0.1% of the total cell protein based on the M_r ~18,000 determined from our earlier PBR purification and cDNA cloning studies (8, 9) and verified here by photolabeling experiments with [^{3}H]PK 14105. This extreme abundance of PBR is comparable with the levels found in adrenal gland mitochondria (6) and is consistent with the high density of these receptors found in apparently all steroidogenic cells (3).

Among the ligands within the series which were tested, PK 11195, PK 14067, and Ro5-4864 exhibited both the highest affinities for PBR and the greatest maximal stimulation of steroid production. Our data reveal only one class of PBR in MA-10 cells, yet the ligands with lower affinities for PBR elicit a lesser maximal stimulation despite the fact that they completely displace [^{3}H]PK 11195 from PBR, with a Hill coefficient of near unity. This implies that the affinity a ligand has for PBR is an important determinant for the magnitude of the steroidogenic response which is achieved. It should also be noted that none of the compounds acted as antagonists, unlike the reports of others (13, 15) examining different functions for PBR. This difference might indicate structural or functional heterogeneity of PBR in different cell systems.

Since hCG produces a very robust stimulation (>300-fold) of steroidogenesis in MA-10 cells (32), a response which is atypical for normal Leydig cells (10-fold stimulation), one might argue that the effects of PBR ligands are very modest and possibly insignificant. This argument was ruled invalid because purified rat Leydig cells also exhibited a significant stimulation of testosterone synthesis in response to PBR ligands. In every respect which we examined, the effects of PBR ligands in purified Leydig cells or their isolated mitochondria were equivalent to the effects observed in MA-10 cells.

For these studies, we used the accumulation of extracellular progesterone as an index of steroid biosynthesis for MA-10 cells (32). The observations that the production of 20α-hydroxyprogesterone, another steroid product of MA-10 cells, was increased at the same level as progesterone and that the metabolism of exogenous pregnenolone and hydrosoluble cholesterol was not altered after addition of ligands indicated that the site of action of PBR ligands was likely to be prior to pregnenolone formation.

Steroid biosynthesis begins with the transport of cholesterol from extramitochondrial stores into mitochondria, where it is metabolized to pregnenolone by P-450$_{scc}$ located on the inner mitochondrial membrane (30, 46, 47). This delivery is the rate-limiting step of steroidogenesis and the main site of action of the acute stimulation by gonadotropin (48). The role of PBR was found to be consistent with its proposed outer mitochondrial membrane localization as PBR ligands had no effect in mitoplasts but did apparently increase cholesterol delivery to P-450$_{scc}$ in intact mitochondria as evidenced by the stimulation of pregnenolone synthesis. More studies are required to elucidate the precise step of the pathway in which PBR function. Based on the studies presented here, PBR could play a role in mitochondrial uptake of cholesterol into a steroidogenic pool and/or transport of cholesterol from the outer to inner mitochondrial membrane. We excluded the possibility that ligand binding to PBR may result in a direct action on P-450$_{scc}$ activity since addition of PBR ligands to Leydig cells did not affect metabolism of (22R)-hydroxycholesterol to progesterone.

When PBR ligands were added together with hCG to MA-10 cells, an additive effect was not seen. This implies that hCG and PBR may act through a common pathway to increase steroid biosynthesis. Since hCG is known to activate multiple cellular events in triggering steroidogenesis, one site of action might include the participation of PBR in the hormone-mediated response. However, since other systems are also affected in the hCG-mediated response, this would account for the differences in kinetics, magnitude of stimulation, and sensitivity to cycloheximide between hCG and PBR ligands.

It had been demonstrated (31, 49, 50) that cycloheximide

blocks hormone-stimulated steroid production and that the inhibition occurs at the site of cholesterol transport into mitochondria. The results in Table I show that cycloheximide does not inhibit ligand-stimulated progesterone production by MA-10 Leydig cells, whereas it essentially abolishes hCG-stimulated steroidogenesis, in agreement with previous results (51). This suggests that promotion of cholesterol transport elicited by PBR ligands is not sensitive to cycloheximide, presumably due to a direct activation of PBR. The kinetics of ligand binding and steroidogenic activity we observed support this proposal. These findings might help account for the suggestions of others (52–54) that hCG-stimulated cholesterol transport is not entirely dependent on a protein synthesis-mediated step. In retrospect, it is also possible that the hormone response may include a cycloheximide-sensitive step to recruit PBR for steroid biosynthesis, and receptor occupancy by ligands merely overrides this physiological control.

In contrast to their relationship with the stimulation by hCG, PBR ligands have an additive effect on steroidogenesis with EGF, another modulator of Leydig cell steroidogenesis (55, 56). It has been reported by Ascoli *et al.* (55) that EGF and submaximal concentrations of hCG stimulate progesterone production by MA-10 Leydig cells using two different pathways. Our results show that PBR ligands and EGF also use different pathways to increase steroid biosynthesis. Moreover, the kinetics of drug action on progesterone production is much more rapid than that of EGF, indicating a more direct action on a regulatory step of steroidogenesis.

While the experiments presented here were in progress, a paper was published by Besman *et al.* (29) showing that diazepam, a ligand for central and peripheral benzodiazepine recognition sites, stimulates pregnenolone formation by bovine adrenal gland mitochondria at a concentration of ~10 μM. Our studies on Leydig cells with diazepam are in agreement with these findings, except that in our model, the EC_{50} of the stimulation obtained with diazepam is ~1 μM. Since we obtained similar values with rat and mouse adrenal gland cells (data not shown), this discrepancy with bovine adrenal gland cells is probably due to the known species differences in receptor affinity for benzodiazepines (57).

In search for physiological endogenous ligands for benzodiazepine receptors, another laboratory at this institution has found that DBI, a polypeptide abundant in steroidogenic tissues, displaces the PBR ligands from rat adrenal gland mitochondrial membranes (25, 26). In addition, the workers found that DBI is expressed in the testis (23, 24), where it primarily localizes in Leydig cells.[2] We have also measured high levels of immunoreactive DBI in MA-10 Leydig cells (data not shown). Furthermore, there are recent reports (28, 29) that a protein from bovine adrenal fasciculata cells which stimulates steroidogenesis was identified as being DBI. These new developments raise the possibility that DBI may interact physiologically with PBR mediating cholesterol delivery into mitochondria, thereby activating steroidogenesis. The uncovering of these potential components in the steroidogenic pathway may prove to be an important step in elucidating the molecular systems which regulate steroid biosynthesis.

Acknowledgments—We are indebted to the following investigators and agencies for the generous gifts of different materials used in these studies: Dr. Mario Ascoli for his generous gift of the Leydig cell line MA-10 and for the helpful instructions on the establishment and use of this cell line; Dr. G. Nieswender for the gift of the 20α-hydroxyprogesterone antisera; Dr. F. F. G. Rommerts for his generous gift of pregnenolone antisera ($gR1\Delta^5$-PR_2); the National Hormone and Pituitary Program (National Institute of Child Health and Human Development, National Institutes of Health) for the gift of hCG; and Dr. C. Gueremy Dr. S. Z. Langer, and Hoffmann-La Roche for the gift of different PBR ligands. We also wish to thank Dr. M. Dym (Georgetown University, Washington, D. C.) for his continuous support of this project.

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Cold Spring Harbor Laboratory Press
1989

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

Printed in the United States of America

9

Book and cover design by Emily Harste

Cover: The electron micrograph of bacteriophage λ particles stained with uranyl acetate was digitized and assigned false color by computer. (Thomas R. Broker, Louise T. Chow, and James I. Garrels)

Cataloging in Publications data

Sambrook, Joseph
Molecular cloning : a laboratory manual / E.F. Fritsch, T. Maniatis—2nd ed.
p. cm.
Bibliography: p.
Includes index.
ISBN 0-87969-309-6
1. Molecular cloning—Laboratory manuals. 2. Eukaryotic cells--Laboratory manuals. I. Fritsch, Edward F. II. Maniatis, Thomas III. Title.
QH442.2.M26 1987
574.87'3224—dc19 87-35464

All Cold Spring Harbor Laboratory Press publications may be ordered directly from Cold Spring Harbor Laboratory Press, 10 Skyline Drive, Plainview, New York 11803. Phone: 1-800-843-4388. In New York (516) 367-8423. FAX: (516) 367-8432.

HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1–3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05 × BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Fraction V; Sigma), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into prehybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The concentration of NaCl is adjusted to 0.1 M, and the solution is extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered, and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD_{260} of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in prehybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6 × SSC or 6 × SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6 × SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

11. *Useful facts*:

    a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5°C + 16.6(\log_{10}[Na^+]) + 0.41(\text{fraction G + C}) - 0.63(\%\ \text{formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[Na^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.

Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.35(\%\ \text{formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.50(\%\ \text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

   The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

Prehybridization solutions

For detection of low-abundance sequences:

Either
6× SSC (or 6× SSPE)
5× Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA

or
6× SSC (or 6× SSPE)
5× Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide

For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

For detection of moderate- or high-abundance sequences:

Either
6× SSC (or 6× SSPE)
0.05× BLOTTO

or
6× SSC (or 6× SSPE)
0.05× BLOTTO
50% formamide

For preparation of BLOTTO, see Table 9.1.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A)$^+$ RNA at a concentration of 1 μg/ml may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of 6 × SSC (or 6 × SSPE) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.

3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane.

   Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C. Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

   When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

Hybridization solution for nylon membranes

6× SSC (or 6× SSPE)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2× SSC and 0.5% SDS at room temperature.

   Important: Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2× SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

   If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1× SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1× SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1× SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

(> 2000 cps on a hand-held minimonitor), hot (> 500 cps on a hand-held minimonitor), and cool (> 50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

13. Cover the filter with a second sheet of Saran Wrap, and expose the filter to X-ray film (Kodak XAR-2 or equivalent) to obtain an autoradiographic image (see Appendix E). The exposure time should be determined empirically. However, single-copy sequences in mammalian genomic DNA can usually be detected after 16–24 hours of exposure at −70°C with an intensifying screen.

Hybridization of Radiolabeled Oligonucleotides to Genomic DNA

Oligonucleotide probes as short as 17 nucleotides in length may be used to detect single-copy sequences in restriction digests of eukaryotic genomic DNA that have been transferred to solid supports. As discussed in Chapter 11, hybrids of this length are stable enough to be detected in practice only if they are perfectly matched. Duplexes with a single base-pair mismatch are significantly less stable and dissociate at a lower temperature than their perfectly matched counterparts (Wallace et al. 1979; Ikuta et al. 1987). It has therefore been possible to use oligonucleotides of defined sequence to probe fetal DNA for the presence of specific point mutations that cause conditions such as sickle-cell anemia (Conner et al. 1983), certain thalassemias (Orkin et al. 1983; Pirastu et al. 1983), and α_1-antitrypsin deficiency (Kidd et al. 1983); to screen DNA extracted from tumor cells for mutations in oncogenes (Bos et al. 1984, 1985, 1987; Forrester et al. 1987; Rodenhuis et al. 1987); and to analyze highly polymorphic loci, for example, the major histocompatibility complex class I genes (Geliebter et al. 1986).

The methods used when hybridizing with oligonucleotide probes are similar to those described earlier in this chapter. However, attention should be paid to the following points:

1. Because of the small size of the target sequence, a minimum of 30 μg of mammalian genomic DNA should be applied to each lane of the agarose gel.
2. The sequences of oligonucleotides used as probes should be long enough to be unique within the target genome (17 nucleotides for the mammalian genome) and short enough to allow the detection of mismatches under the conditions of hybridization used. Typically, oligonucleotides used for screening mammalian genomic DNA are 19–21 nucleotides in length.
3. When used to detect point mutations, oligonucleotides are used in pairs; one member of the pair is perfectly homologous to the mutated gene sequence and the other is homologous to the wild-type sequence. Usually, the members of the pair differ in sequence by only one nucleotide. Before embarking on an analysis of genomic DNA with these probes, it is essential to establish hybridization and washing conditions using cloned fragments of DNA of known sequence that are homologous to each member of the pair of oligonucleotides. These methods are discussed in detail in Chapter 11. Reconstruction experiments, in which known amounts of the control DNAs are added to a large excess of genomic DNA (at least 30 μg), are then used to test the sensitivity of the system.
4. Oligonucleotides are radiolabeled by [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase (see Chapter 11). These probes tend to hybridize nonspecifically to high-molecular-weight DNA immobilized on nitrocellulose filters or nylon membranes, producing a smear toward the top of the autoradiograph. It is therefore important to choose a restriction enzyme (or a combination of restriction enzymes) that yields a hybridizing fragment whose size is not greater than 5 kb.

5. After electrophoresis, the fragments of genomic DNA may be transferred to a solid support by the conventional Southern transfer technique or immobilized within the agarose gel itself by dehydration (Studencki and Wallace 1984). Although DNA immobilized within the gel appears to give somewhat stronger hybridization signals than DNA attached to a solid support, it cannot be hybridized sequentially to many different probes. This is a severe disadvantage when the amount of genomic DNA is limited (as is often the case in prenatal diagnosis, for example). We therefore recommend that the genomic DNA be transferred to a nylon membrane such as Nytran (Schleicher and Schuell) or GeneScreen (du Pont).

6. Wherever possible, negative and positive hybridization controls should be included in each gel.

7. Oligonucleotides may also be used to detect rare transcripts in northern blots that contain 30 μg of total cellular RNA (Zeff et al. 1986) or 5 μg of poly(A)$^+$ RNA (Geliebter et al. 1986).

Removal of Radiolabeled Probes from Nitrocellulose Filters and Nylon Membranes

Probes become irreversibly bound if nitrocellulose filters and nylon membranes are allowed to dry. Therefore, every effort should be made to ensure that the solid supports remain wet at all stages during hybridization, washing, and exposure to X-ray film.

REMOVING PROBES FROM NITROCELLULOSE FILTERS

1. Heat several hundred milliliters of 0.05 × SSC, 0.01 M EDTA (pH 8.0) to boiling. Remove the fluid from the heat and add SDS to a final concentration of 0.1%. Immerse the filter in the hot elution buffer for 15 minutes.

2. Repeat step 1 with a fresh batch of boiling elution buffer.
   Important: Do not allow the filter to dry when transferring it between batches of hot elution buffer.

3. Rinse the filter briefly in 0.01 × SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

4. Sandwich the damp filter between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.

5. The filter may now be dried, wrapped loosely in aluminum foil, and stored under vacuum at room temperature until needed.

REMOVING PROBES FROM NYLON MEMBRANES

1. Either
   - immerse the membrane in several hundred milliliters of 1 mM Tris · Cl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1 × Denhardt's reagent (see Table 9.1) for 2 hours at 75°C, or
   - immerse the membrane in 50% formamide, 2 × SSPE for 1 hour at 65°C.

2. Rinse the membrane briefly with 0.1 × SSPE at room temperature. Remove most of the liquid from the membrane by placing it on a pad of paper towels.

3. Sandwich the damp membrane between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.

4. The membrane may now be dried, wrapped loosely in aluminum foil, and stored under vacuum at room temperature until needed.

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